PRESENCE AND FUNCTIONAL ROLE OF THE POLYPEPTIDE GROWTH FACTORS INSULIN-LIKE GROWTH FACTOR I (IGF-I) AND EPIDERMAL GROWTH FACTOR (EGF) IN THE REPTILIAN OVIDUCT

By

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To Mom, Dad, and Beth,

for all your love, support, and guidance.

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PRESENCE AND FUNCTIONAL ROLE OF THE POLYPEPTIDE GROWTH FACTORS INSULIN-LIKE GROWTH FACTOR I (IGF-I) AND EPIDERMAL GROWTH FACTOR (EGF) IN THE REPTILIAN OVIDUCT

By

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Insulin-like growth factor I (IGF-I) and epidermal growth factor (EGF) are polypeptide growth factors involved in uterine proliferation and differentiation and embryonic development in mammals. The purpose of this study was to examine the presence and function of these two growth factors in reptiles. Positive immunostaining for IGF-I-like and EGF-like material in the oviduct of a turtle, lizard, alligator, and tuatara using heterologous polyclonal antibodies suggests a high degree of homology between the reptilian forms of these growth factors and their mammalian counterparts. Likewise, it suggests a role for these growth factors in reptilian reproduction.

Seasonal development of the oviduct in reptiles, as in all vertebrates, is regulated primarily by estradiol-17β. Rather than acting directly, however, estradiol-17β appears to exert its proliferative and differentiative effects via intermediate growth factors. To test the hypothesis that IGF-I and EGF mediate estrogen-induced oviductal growth and differentiation in the reptilian oviduct, ovariectomized Mediterranean geckos, *Hemidactylus turcicus*, were treated with estradiol-17β, IGF-I, or EGF, and their oviducts were examined histologically. Both IGF-I and EGF had a pronounced stimulatory effect on epithelial and glandular growth in the absence of estradiol-17β.

In addition to the possible role of IGF-I in the proliferation and differentiation of the reptilian oviduct, the presence of IGF-I-like immunoreactivity at the apical tips of the epithelial cells and in the endometrial glands of the oviductal tube suggests IGF-I is incorporated into the egg albumen and could play a critical role in embryonic development. Yolk and albumen samples from the eggs of an American alligator *Alligator mississippiensis* were analyzed for IGF-I by specific radioimmunoassay, and IGF-I was present in both with albumen IGF-I concentrations being approximately tenfold greater than those found in yolk. The presence of both IGF-I and EGF in reptiles suggests a central role for these growth factors in normal cellular growth and differentiation, and tissue morphogenesis.

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

Reptilian Oviductal Anatomy

In reptiles, as in all tetrapod vertebrates, the oviducts are derived embryonically from the paired Müllerian ducts (Hildebrand, 1982). The term "oviduct" as defined in comparative anatomical studies and as used throughout this dissertation refers to the entire female reproductive tract. Although the gross morphology of the reptilian oviduct has been described (Fox, 1977; Palmer and Guillette 1989; Guillette et al., 1989), the functional anatomy of this organ is still poorly understood. Considerable variation exists in oviductal morphology among reptilian species. The reptilian oviduct is typically divided into four major functional regions: infundibulum, tube, uterus, and vagina (Cuellar, 1966; Fox, 1977). The most anterior portion of the oviduct, the infundibulum, functions to receive eggs from the ovaries (Cuellar, 1970) and is presumably the site of fertilization. The tube region is the site of albumen production, whereas the uterus is involved in secreting the fibrous membrane and the calcium coat of the eggshell as well as retaining the eggs prior to oviposition. The vagina is a short segment of the reptilian oviduct which connects the uterus to the cloaca. The tuatara (*Sphenodon punctatus*) exhibits the most primitive mode of eggshell formation. Both the fibrous layer and the calcareous layer of the eggshell are produced simultaneously by the uterus. Turtles and squamates, in contrast, have a temporal separation of these two processes. Both fibers

and calcium are laid down by the uterus, but these steps occur sequentially. The crocodilians exhibit the most structurally and functionally advanced oviduct among the reptiles, and they possess a uterus which is divided into an anterior fiber-secreting region and a posterior calcium-secreting region. Interestingly, this oviductal anatomy is similar to that which is seen in birds. The tube, fiber uterus, and calcium uterus of the alligator oviduct are structurally and functionally homologous to the avian magnum, isthmus, and shell gland respectively.

Endocrinology of Vertebrate Reproduction

In all vertebrates, reproduction is regulated through the hypothalamo-hypophysial axis which coordinates gonadal function through the regulation of circulating gonadotropins (Fig. 1-1). The ovary has a dual function: to produce ova and to synthesize steroid hormones. Cyclic ovarian development involving the proliferation and differentiation of growing follicles has classically been viewed as regulated by the gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Although the highly coordinated hormonal activity responsible for follicular growth and development is still under investigation, it is clear that these gonadotropins in addition to estradiol-17β (E_2) are essential components of the system.

The release of FSH and LH from the anterior pituitary gland appears to be under the control of a single hypothalamic hormone, gonadotropin-releasing hormone (GnRH). FSH stimulates gametogenesis and steroidogenesis, whereas LH is primarily responsible for controlling steroidogenesis. The gonadal steroids (principally, estradiol-17β, progesterone, and testosterone) are released from the ovary in response to the gonadotropins. Circulating steroids normally inhibit the release of more

GnRH through a negative feedback mechanism. Circulating steroids also produce a negative effect on the release of FSH and LH from the anterior pituitary. An additional factor produced by the ovary, inhibin, is also involved in the inhibition of FSH release.

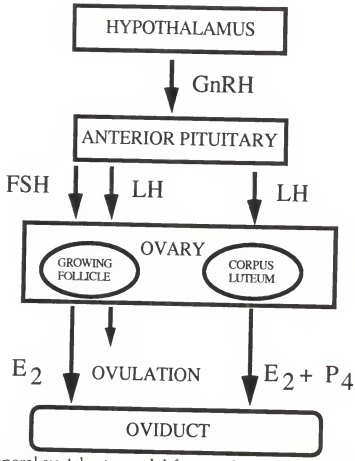

Figure 1-1. General vertebrate model for regulation of gonadal function.

In reptiles, ovarian function is also controlled by gonadotropin secretion from the pituitary gland (see Licht, 1984, for review). Gonadotropin release is regulated by GnRH. Most reptiles produce two distinct gonadotropins, FSH and LH although the squamates (lizards and snakes) appear to possess only a single FSH-like gonadotropin. The major circulating

estrogen in nonmammals is estradiol-17β (E_2). Other important gonadal steroids, as in mammals, are progesterone (P_4) and testosterone (T).

In addition to these hormones, local autocrine/paracrine growth factors such as the insulin-like growth factors (Savion et al., 1981; Baranao and Hammond, 1984; Li et al., 1983), epidermal growth factor (Gospodarowicz and Birdwell, 1977; Gospodarowicz and Bialecki, 1979), fibroblast growth factor (Gospodarowicz and Birdwell, 1977; Gospodarowicz and Bialecki, 1979), transforming growth factor-α and transforming growth factor-β, and angiogenic factors have been hypothesized to play a role in follicular recruitment and development. The ovarian granulosa cell is a site of insulin-like growth factor-I (IGF-I) secretion and action, suggesting an autocrine role for this polypeptide growth factor in the porcine ovary (Hammond et al., 1985). The major trophic hormones implicated in follicular growth (e.g., FSH, LH, and E_2) do not stimulate mitosis in cultured porcine granulosa cells whereas insulin, IGF-I, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and ovarian follicular fluid were potent stimulators of DNA synthesis and mitosis (Hammond and English, 1987). Similarly, the mitogenic effects of EGF and fibroblast growth factor (FGF) on cultured granulosa cells of rodent, porcine, and human origin have been demonstrated (Gospodarowicz and Bialecki, 1979).

Hsu and Hammond (1987) studied the effects of FSH, LH, and E_2 on the secretion of immunoreactive IGF-I by cultured porcine granulosa cells and observed that gonadal IGF-I secretion could be stimulated by these hormones. Human luteal cells of granulosa origin appear to require the early exposure and continuous presence of EGF for their stimulatory effect on P_4 production (Serta and Seibel, 1993). EGF stimulates mitosis in cultured human granulosa cells and is a potent inhibitor of aromatase, thus reducing

the synthesis of estrogens. In contrast, the IGFs are also mitogenic to ovarian granulosa, but are potent stimulators of aromatase and granulosa E_2 production (Guidice et al., 1993). Cyclic follicular development is a complex process and the role of polypeptide growth factors in this process is now a major focus of research on mammalian ovarian function.

Hormonal regulation of the development of the reptilian oviduct, as in other vertebrates, involves the steroid hormones E_2 and P_4. For example, during follicular development in the alligator, ovarian follicles increase in size and secrete E_2 in response to pituitary gonadotropin secretion (Lance, 1989). Estradiol-17β secreted by the developing follicles serves two basic functions. First, E_2 stimulates the liver to synthesize the yolk precursor protein vitellogenin, which is then secreted into the blood, carried to the ovaries, and taken up by the oocytes, and transformed into yolk (Ho et al., 1982). Second, E_2 stimulates growth and differentiation of the reproductive tract in preparation for oviposition (Mead et al., 1981; Jones and Guillette, 1982; Guillette et al., 1991). Uterine cellular growth and differentiation can be stimulated *in vivo* by E_2. Yaron (Yaron, 1972) demonstrated that both E_2 and P_4 are essential for the maturation of the genital tract in the preovulatory stage in the viviparous lizard, *Xantusia vigilis*. Additional studies have demonstrated that treatment of oviducts with exogenous E_2 stimulates hypertrophy of oviductal glands (Christiansen, 1973; Fawcett, 1975). Ortiz and Morales (1974) demonstrated that ovarian tissue was capable of stimulating new gland formation from luminal epithelia in oviductal tissue cultured *in vitro*. Mead et al (1981) studied the effects of P_4 and E_2 on the histology of the oviduct of the garter snake *Thamnophis elegans* and reported that E_2 was the most important ovarian hormone in regulating development of the uterus. They also concluded that additional hormones other than P_4 may be required

for full restoration of the garter snake uterus to preovulatory condition since administration of E_2 alone or in combination with P_4 to ovariectomized snakes failed to completely restore the uterus to such a state.

The mechanisms by which steroid hormones such as E_2 elicit their responses in target tissues is not completely understood. The uterine response to E_2 involves proliferation of a wide variety of cell types with the most significant effects on the epithelium and underlying stroma (Kirkland et al., 1979). The E_2 response in the uterus can be divided into two distinct stages. The first stage occurs within approximately 4 hours of E_2 administration and involves an increase in uterine weight (Mueller et al., 1958), glucose metabolism (Meier and Garner, 1987), histamine depletion (Szego, 1959), and RNA polymerase activity (Aziz et al., 1979). Also during this earliest stage of the E_2 response, enhancement of growth factor expression occurs (Mukku and Stancel, 1985). The later stage of the E_2 response, which occurs approximately 4 to 48 hours following E_2 treatment includes increase in uterine dry weight and DNA content (Kaye et al., 1972; Quamby and Korach, 1984). Although the biological actions of E_2 resulting in cellular hypertrophy and hyperplasia have been identified, the specific molecular mechanisms involved in E_2 action remain to be elucidated.

Recent studies have led to the hypothesis that these hormones regulate cellular growth and differentiation by stimulating the biosynthesis of peptide growth factors which act in an autocrine or paracrine fashion to mediate steroid hormone actions. While an estrogen response is easily elicited *in vivo*, it has been difficult to elicit such a response in isolated uterine cells *in vitro*, thus leading to the hypothesis that locally derived paracrine factors could be involved in mediating the E_2 response. The observation that stromal tissue is necessary for estrogen responsiveness supports the theory

that paracrine growth factors are involved in the mediation of estradiol-induced cellular growth and differentiation (Cooke et al., 1986; Cunha and Young, 1992). Further support for this theory comes from the observation that estrogen stimulates DNA synthesis in murine uterine epithelial cells which lack estrogen receptors (Bigsby and Cunha, 1986). This intermediate role for growth factors was demonstrated clearly in recent studies showing that epidermal growth factor (EGF) was capable of eliciting most of the effects attributed to estrogens in ovariectomized mice (Nelson et al., 1991). Further, treatment of mice with anti-EGF abolished most of the estrogenic response in the reproductive tract.

Although EGF clearly mediates many estradiol-induced responses in the reproductive tract, other growth factors have been isolated from reproductive tract tissue. The transforming growth factors (TGFα and TGFβ), insulin-like growth factors (IGF-I and II), platelet-derived growth factor (PDGF), and several heparin-binding growth factors (HBGFs) have been suggested as possible paracrine regulators of endometrial cell proliferation and differentiation. Nelson et al. (1992) concluded that in the mouse uterus, TGFα is estrogen inducible, TGFα antibody inhibits uterine growth, and functional TGFα/EGF receptors are present. IGF-I has been implicated as one of the mediators of estradiol-induced proliferation of the female reproductive tract (Murphy and Friesen, 1989). Expression of both IGF-I and its receptor in the uterus appear to be responsive to E_2 (Murphy and Friesen, 1988; Ghahary and Murphy, 1989). Within one hour of administration of a single injection of E_2 to pituitary-intact ovariectomized rats, a significant increase in uterine extractable IGF-I was seen. Additionally, an increase in uterine IGF-I mRNA was observed following E_2 treatment. These data suggest that multiple

polypeptide growth factors play important roles in the estrogen-induced response of the oviduct.

Growth Factors

Epidermal Growth Factor (EGF) System

Epidermal growth factor

Epidermal growth factor, a single-chain polypeptide (MW 6045), was first isolated from the submaxillary glands of adult male mice (Cohen, 1962). EGF was subsequently isolated from human urine and found to share 70% amino acid sequence homology with mouse EGF (Cohen and Carpenter, 1975; Gregory, 1975) and was thought to be identical to urogastrone, a potent inhibitor of gastric acid secretion. Mouse EGF and human EGF/urogastrone compete for the same membrane receptors and elicit the same biological responses (Hollenberg and Gregory, 1977). EGF has now been characterized in the milk of humans (Carpenter, 1980; Shing, 1984), rats (Berseth et al., 1983; Berseth, 1987), and mice (Hirata and Orth, 1979) as well as other biological fluids such as plasma (Gregory, 1975; Gregory and Preston, 1977), amniotic fluid (Starkey and Orth, 1977), and colostrum (Brown and Blakely, 1984). EGF was first described as a mitogen for epidermal cells (Cohen and Savage, 1974) and was later shown to stimulate fibroblast proliferation (Cohen and Carpenter, 1975). Gospodarowicz and Birdwell (1977) demonstrated that EGF serves as potent mitogen for cultured bovine ovarian cells. EGF also stimulates smooth muscle cells of calf aorta and human uterus (Carpenter and Cohen, 1979; Bhargava et al., 1979).

Epidermal growth factor is one of the polypeptide growth factors which has been implicated in estrogen-induced uterine growth. All cell types of the

human uterus (stromal cells, glandular epithelium of the endometrium, elongated and circular muscle cells of the myometrium, smooth muscle and endothelial cells of arterioles in the basal endometrium and myometrium) contain EGF binding sites and stromal cells contain the highest number of EGF-binding sites (Chegini et al., 1986). Several laboratories have shown that EGF is present in the mouse uterus (Nelson et al., 1991; DiAugustine et al., 1988; Huet-Hudson et al., 1990) and that EGF and EGF receptors are enhanced by E_2 (DiAugustine et al., 1988; Huet-Hudson et al., 1990; Gardner et al., 1989; Mukku and Stancel, 1985; Paria and Dey, 1990). EGF acts as a potent mitogen for cultured uterine epithelial cells (Tomooka et al., 1985). A study with ovariectomized mice (Nelson et al., 1991) has shown that EGF can mimic estrogen-induced stimulation of reproductive tract growth and differentiation. Snedeker et al. (1991) have shown that EGF can stimulate normal mammary gland ductal growth in mice in the absence of ovarian steroids providing further evidence that EGF is a mediator of E_2 action.

Conflicting data have been reported concerning the role of EGF in preimplantation embryo development in mice. Paria and Dey (1990) found that although TBFβ and IGF-I enhance embryo development, the response is not as great as shown by EGF alone. Goldman et al. (1993) reported that 100ng/ml EGF inhibited the growth of 4-cell embryos into blastocysts and the development of blastocysts to the attachment and spreading stages. Other authors (Caro et al., 1987; Wood and Kaye, 1989) have reported that EGF-supplemented media did not enhance embryonic development *in vitro*. These contradictory results are likely due to different culturing methods. Nevertheless, these studies underline the difficulties of studying the physiological effects of polypeptide growth factors due to their ubiquitous presence and their complex interactive mechanisms of action.

Evolution

Little information is available concerning EGF in non-mammalian species. Shetty et al. (1992) isolated and purified a growth factor from pigeon milk (a nutritive crop secretion of pigeons and doves that is fed to their young during the early postnatal period) and reported that this was the first evidence of an EGF-like protein in a non-mammalian secretion. Later studies (Shetty and Hegde, 1993) of pigeon milk-derived growth factor (PMGF) revealed that the biological actions of this growth factor resemble those reported for EGF, primarily the ability to cause precocious incisor eruption and eyelid opening in new-born mice (Cohen, 1962). Epidermal growth factor stimulates mitosis in chick embryo epithelial cells (Cohen, 1965) and EGF receptors have been found in chick tissues (Santora et al., 1979) providing further evidence for the presence of EGF-like molecules in non-mammalian species.

Insulin-like Growth Factor (IGF) System

IGF-I is a unique hormone in that its biological actions can be elicited in autocrine, paracrine, and endocrine fashion. The IGF system consists of the IGF peptides (IGF-I and IGF-II), their receptors (type I and type II), and a family of proteins known as IGF binding proteins (IGFBPs) which regulate the availability of the IGFs to their target cells.

IGF peptides

Insulin, IGF-I and IGF-II are members of the same family of regulatory peptides, share a high degree of sequence homology, exhibit several common biological functions, and exhibit receptor cross-reactivity. Whereas insulin was first discovered by Banting (1921) and its amino acid sequence was determined by Sanger (1955), the IGFs were first discovered as a result of three

distinct biological activities of serum: sulfation factor activity (SFA), nonsuppressible insulin-like activity (NSILA), and multiplication-stimulating activity (MSA). While Salmon and Daughaday (1957) were searching for an *in vitro* bioassay system for growth hormone, they observed that growth hormone itself had no effect on cartilage metabolism, but that the plasma from normal rats stimulated sulfate uptake, a principal physiological phenomenon in cartilage growth. These observations led to the proposal that growth hormone itself does not stimulate growth processes *in vitro* and *in vivo*, but rather that it stimulates the production of "factors" that mediate the message of growth hormone. Another important observation was made around this time. After the radioimmunoassay for insulin was developed, it became apparent that serum contained far more insulin-like activity than could be attributed to the insulin content of the serum alone. This insulin-like activity was called NSILA by Froesch et al. (1963). A third observation which led to the discovery of the IGFs involved the search for the components of serum which were necessary for the growth of cells in culture. Temin and others demonstrated in the 1960s that cellular proliferation in some cell lines was dependent on the presence of certain factors in serum whereas cells in other lines produced their own growth-stimulating substances. Multiplication-stimulating activity (MSA) was one such activity isolated from the medium of cultured rat liver cells. It later became apparent that all three of these biological activities were the result of similar, if not the same, substances. In 1972, the term somatomedin was introduced to denote the then uncharacterized factor that mediated the action of growth hormone in the stimulation of somatic growth and that displayed insulin-like activity. Later, NSILA was purified and two biologically active peptides, IGF-I and IGF-II, were characterized and their primary structure determined (Rinderknecht

and Humbel, 1976; Rinderknecht and Humbel, 1978a; Rinderknecht and Humbel, 1978b). It has become apparent during the past decade that only IGF-I is regulated by growth hormone; consequently, the term somatomedin is no longer adequate to describe both IGF-I and IGF-II. Thus, in 1987, the term IGFs was recommended to refer to this family of peptides (Daughaday et al., 1987).

IGF-I and IGF-II were isolated from adult human plasma and characterized by Rinderknecht and Humbel (1978a, 1978b). IGF-I and IGF-II are single-chain molecules with three intrachain disulfide bridges consisting of 70 (MW 7,646) and 67 (MW 7,471) amino acids respectively. Human IGF-I and IGF-II exhibit an amino acid sequence homology of 62%. They each consist of an A-domain homologous to the A-chain of insulin and a B-domain homologous to the B-chain of insulin. In contrast to insulin, however, the C-domain is retained in IGF-I and IGF-II and consists of 12 and 8 amino acids respectively. In addition to this C-domain, the IGFs contain a carboxy-terminal D-domain. In humans, the gene coding for IGF-I is located on the long arm of chromosome 12 (Rotwein, 1986), and the gene coding for IGF-II is on the short arm of chromosome 11, which also contains the human insulin gene (Bell, 1985). The human IGF-I gene (45 kilobases) consists of six exons, five introns, and at least two promoters (see Rotwein, 1991, for review). Exons 1 and 2 code for the amino terminus of the signal peptide. Exons 3 and 4 code for the mature 70 amino acid peptide in addition to part of the signal peptide and the proximal portion of the E peptide. Exons 5 and 6 code for the carboxy-terminal regions of the E peptide. The structural complexity of the IGF-I gene allows for regulation of gene expression at several levels in the biosynthetic pathway including transcription, RNA processing and transport, mRNA stability, translation, and post-translational events (LeRoith, 1991). Two major classes of IGF-I mRNA transcripts have

been identified in hepatic and nonhepatic tissues (LeRoith, 1991). These transcripts are different due to different transcription starts in exon 1 and exon 2. Exon 2 transcripts are regulated primarily by GH and have been proposed to encode the "endocrine" form of IGF-I. Exon 1 transcripts, on the other hand, are regulated by factors other than GH and may represent the "autocrine/paracrine" form of the peptide.

These two mRNAs result from different transcription starts in exon 1 and exon 2. Exon 2 transcripts are hypothesized to be the endocrine form of IGF-I and exon 1 transcripts, in contrast, are thought to be the local autocrine/paracrine form of the peptide. Exon 2 transcripts are primarily regulated by GH, whereas exon 1 transcripts are regulated by factors other than GH. Differential splicing of the primary transcripts results in either IGF-Ia or IGF-Ib mRNAs. Consequently, the human IGF-I gene encodes for two different protein precursors: one containing 153 amino acids, including an E peptide of 35 amino acids (IGF-Ia) and another containing 195 amino acids, including an E peptide of 77 amino acids (IGF-Ib). Two different forms of the final protein product, truncated and intact, have been identified. Sara and Hall (1990) have proposed that the truncated peptide is the local form of IGF-I, and the intact, full-length form is the endocrine form of the peptide. They also speculate that the two forms of the final peptide are related to the alternative primary IGF-I transcripts that encode for the two different protein precursors, IGF-Ia and IGF-Ib. Specifically, because IGF-Ia mRNA predominates in tissues and IGF-Ib mRNA has been identified as the hepatic source of circulating IGF-I, the truncated form of the mature peptide may be translated from the IGF-Ia mRNA transcript and the intact form may be translated from the IGF-Ib mRNA transcript. The proposed final step for synthesis of the two mature proteins (truncated and intact) involves post-

translational cleavage of the E domains. According to this hypothesis, the binding site for the enzyme which releases the truncated IGF-I is present only on the IGF-Ia precursor protein. This hypothesis provides an explanation for the necessity of two peptide precursors for IGF-I. The biological activities resulting from the alternative forms of IGF-I have yet to be determined. The complexity of the regulation of the IGF-I gene provides many levels at which IGF-I expression can be controlled.

IGF receptors

The IGF peptides bind to their receptors and activate an endogenous tyrosine-specific protein kinase and autophosphorylation of the receptor further enhances the kinase activity which results in the phosphorylation of cellular proteins. These activated proteins serve as intermediate messengers in the cascade of events leading to cellular responses. There are two IGF receptors: the type I receptor (which preferentially binds IGF-I) and the type II receptor (which preferentially binds IGF-II). The structure of the IGF-I receptor is closely related to that of the insulin receptor. Both are tetramers consisting of two extracellular α-subunits (~130Kd) and two transmembranal β–subunits (~95Kd) which are linked by interchain disulfide bonds (Ullrich et al., 1986). The binding of the ligand to the α-subunit results in intracellular signal transduction by the autophosphorylation of tyrosine residues within the intracellular β-subunit. The IGF-II receptor is distinct from the IGF-I and insulin receptors. It has a high affinity for IGF-II, a low affinity for IGF-I, and does not bind insulin. The IGF-II receptor is a single chain glycoprotein with 90% of its linear structure being extracellular (Morgan et al., 1987). It has no intrinsic tyrosine kinase activity, but the small cytoplasmic domain contains several potential phosphorylation sites on tyrosine, threonine, and serine residues. Complementary DNA analysis has revealed that the IGF-II receptor

is almost identical to the mannose-6-phosphate receptor which functions mainly as a lysozymal enzyme targeting protein. Many of the mitotic and growth-promoting effects of IGF-II appear to be mediated via the IGF-I receptor in addition to the IGF-II receptor. Regardless of the ligand which binds to a receptor, the resultant cellular response is that characteristic of the receptor. The fact that both ligands (IGF-I and IGF-II) can activate both receptors (type I and type II) generates uncertainties when attempting to interpret experimental data.

Interestingly, some tissues express hybrid receptors composed of one insulin receptor alpha and beta subunit and one IGF-I receptor alpha and beta subunit. In placenta, 70% of the receptors are estimated to be hybrids (Soos et al., 1990). This hybrid receptor has a higher affinity for IGF-I than for insulin and IGF-I is more effective in activating autophosphorylation; therefore, the hybrid receptor acts more like an IGF-I receptor than an insulin receptor. This receptor "cross-talk" makes data interpretation difficult.

IGFBPs

The IGFs are found complexed with binding proteins in serum, amniotic fluid, and in conditioned media from a variety of human cell types. These binding proteins (IGFBPs) have a number of potential roles in the IGF system. First, IGFBPs inhibit the mitogenic effects of the IGFs by limiting the access of the peptides to cell surface receptors. IGFBPs have also been shown to potentiate the actions of IGFs. They may do this by protecting them from degradation and thus prolonging their half-lives (Ballard et al., 1991; Guler et al., 1989), by facilitating transport of the peptides to target cell, or by serving as a storage reservoir for the IGFs that allow for the controlled delivery of the peptides to their target cells. Circulating levels of IGFBPs in serum are

regulated by various hormones, nutritional status, and proteases (see Cohick and Clemmons, 1993, for review).

The complete primary structures of six IGFBPs in humans have been determined and designated IGFBP-1 through 6 (see Shimasaki et al., 1992, for review). Each of the 6 IGFBPs consist of 200-300 amino acids. These six IGFBPs are often divided into two classes: the growth hormone-dependent main serum IGFBP (IGFBP-3) and the lower molecular weight, growth hormone-independent IGFBPs. IGFBP-1 is the main IGFBP in amniotic fluid and was first identified as a placental protein, placental protein-12 (Koistinen et al., 1986). IGFBP-1 binds IGF-I and IGF-II with approximately equal affinity. The cDNA sequences of human and rat IGFBP-1 are 58% homologous (McCusker and Clemmons, 1992). Complete structures of human, rat, and bovine IGFBP-2 have been determined from cDNA sequence analysis (Bourner et al., 1992; McCusker and Clemmons, 1992). The affinity of IGFBP-2 for IGF-II is threefold higher than for IGF-1. IGFBP-3 is the major IGFBP in adult serum. It circulates as a 150Kd complex composed of IGFBP-3 (β-subunit), an acid-labile (α) subunit, and IGF (γ-subunit) (Baxter and Martin, 1989). IGFBP-3 has a very high affinity for both IGF-I and IGF-II and binds greater than 95% of the IGF-I and IGF-II in circulation. IGFBP-4 has been isolated from human serum (Binoux et al., 1982) and the conditioned media of a human breast cancer cell line (DeLeon et al., 1988), fibroblasts (Conover et al., 1989), and osteoblasts (Mohan et al., 1989). IGFBP-4 has a high affinity for IGF-I and IGF-II that is nearly equal to that of IGFBP-3 at pH 7.4. IGFBP-5 has been isolated from human, porcine, and rat sources (Andress and Birnbaum, 1991; Bautista et al., 1991; Shimasaki et al., 1991a). IGFBP-5 has the highest affinity for IGF-I and IGF-II of all the IGFBPs. IGFBP-6 has been purified from porcine follicular fluid (Shimasaki et al., 1991b) and several human sources,

including serum, cerebrospinal fluid, and the conditioned media of fibroblasts and osteosarcoma cells (Forbes et al., 1990; Martin et al., 1990; Zapf et al., 1990; Roghani et al., 1991; Andress and Birnbaum, 1991).

In nonmammalian vertebrates, IGFBPs have been identified in the toads, *Bufo woodhousei* (Pancak-Roessler, 1990) and *Bufo arenarum* (Cortizo, 1993); the coho salmon, *Oncorhynchus kisutch*, the striped bass, *Morone saxatilis*, tilapia, *Oreochromis mossambicus*, and the longjawed mudsucker, *Gillichthys mirabilis*, (Kelley et al., 1992); and the golden perch, *Macquaria ambigua* (Anderson et al., 1993).

IGF-I regulation

Many hormones have been implicated as regulators of IGF-I gene expression in various target tissues. The regulation of circulating concentrations and tissue synthesis of IGF-I by GH is well documented (Baxter, 1989; Daughaday, 1989). This GH-regulated expression of the IGF-I gene appears to be developmentally dependent because GH does not stimulate fetal IGF-I production presumably because of a lack of mature GH receptors in the fetus. High glucocorticoid levels have been associated with a decrease in IGF-I activity. However circulating levels of IGF-I remain normal leading to the conclusion that these hormones inhibit the biological activites of IGF-I rather than inhibiting expression of the IGF-I gene (Gourmelen et al., 1982; Furlanetto et al., 1977; Unterman and Phillips, 1985). Contradictory effects of E_2 on IGF-I have been described. Pubertal increases in circulating IGF-I are seen in both primates (Copeland et al., 1984; Rosenfield et al., 1983) and rats (Handelsman et al., 1987). This correlation between E_2 and IGF-I is further supported by experimental data in which girls with Turner's syndrome and thus depressed circulating E_2 levels (Ross et al., 1983) and boys (Cassorla et al., 1984) treated with variable doses of E_2 showed significant

increases in IGF-I. A similar stimulatory effect of E_2 on IGF-I has not been shown in adult humans. No E_2-related IGF-I variation has been detected during the normal menstrual cycle. Estradiol-17β replacement therapy has been shown to decrease IGF-I levels in postmenopausal women (Duursma et al., 1984) and in normal men (Caufriez et al., 1986). Therefore, it is apparent that the effects of E_2 on IGF-I vary based on developmental stage and other hormonal interactions. Murphy and Friesen (1988) demonstrated that both E_2 and GH are potent stimulators of IGF-I gene expression in appropriate target tissues and that in addition to any effects that E_2 has on GH secretion and IGF-I production, the growth-retarding effect of E_2 in the rat involves inhibition of GH-dependent hepatic IGF-I expression. In addition to E_2, other gonadal steroids influence IGF-I synthesis and biological activity. A significant increase in IGF-I has been associated with the high-P_4 phase of the menstrual cycle in women (Caufriez et al., 1986). During puberty, an increase in IGF-I has also been associated with elevated testosterone levels (Rosenfield et al., 1983).

Many hormones and other polypeptide growth factors appear to be able to regulate paracrine IGF biosynthesis. Johnson et al. (1988) concluded that both GH and insulin are involved in the accumulation of IGF-I mRNA transcripts and in the secretion of IGF-I from cultured rat liver hepatocytes. Clemmons and Shaw (1983) demonstrated that platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) could stimulate IGF-I production from human fibroblasts *in vitro*. Recently, it has been shown that many trophic hormones in addition to their classic action on secondary hormone production can also stimulate IGF-I production and secretion from their target tissues. In the granulosa cells of the ovary and in the Sertoli cells of the

testis, for example, IGF-I production is stimulated by FSH and LH (Hammond et al., 1985).

Evolution

The IGFs are members of a superfamily of insulin-like hormones which include insulin (Steiner et al., 1989) and relaxin (Hudson et al., 1983) in the vertebrates and the silkworm hormone bombyxin (Adachi et al., 1989), insulin-related peptide from the locust, *Locusta migratoria* (Lagueux et al., 1990), insulin-related molecules in the sea urchin, *Strongylocentrotus purpuratus*, (De Pablo et al., 1988), molluscan insulin-like peptide, from *Lymnaea stagnalis*, (Smit et al., 1988), and prothoraciotropic hormone in the insect *Manduca sexta* (Iwami et al., 1989) in invertebrates. Insulin activity has also been reported in extracts of blowflies (Duve et al., 1979; LeRoith et al., 1981). Sponges contain molecules which are structurally similar to human insulin (Robitzki et al., 1989). The discovery of insulin-related peptides in these invertebrates supports the hypothesis that insulin arose more than a billion years ago and therefore has a very early phylogenetic origin. The origin and evolutionary relationship between insulin and the IGFs is less clear.

Chan et al. (1990) cloned and sequenced the cDNA coding an insulin-like peptide (ILP) from the primitive cephalochordate, *Branchiostoma californiensis*, and reported that the deduced amino acid sequence for prepro-ILP indicates that it has characteristics of a hybrid insulin/IGF molecule. This hybrid molecule may represent a transitional form connecting insulin and IGF. The organization and similarity of the amphioxus insulin-like peptide (ILP) gene to vertebrate insulin genes and the sequence similarity between ILP and the IGFs provide the basis for the hypothesis that IGF could have

emerged from an ancestral two-chain insulin molecule early in vertebrate evolution.

The Atlantic hagfish, *Myxine glutinosa*, a primitive vertebrate species which is believed to have evolved directly from cephalochordates or some cephalochordate-like ancestor, diverged from the main line of vertebrate evolution approximately 550 million years ago and thus occupies a key branch point on the evolutionary tree. The prepro-IGF amino acid sequence for the hagfish contains A and B domains which are equally homologous to human prepro-IGF-I (35 out of 50 amino acids) and prepro-IGF-II (37 out of 53 amino acids) (Nagamatsu et al., 1991). Chan et al. (1981) have shown that the hagfish also possesses a highly conserved insulin gene that is expressed in a discrete islet organ located near the intestine suggesting that the split between insulin and IGF genes occurred prior to the divergence of the jawless fishes from the main vertebrate line.

The genes for IGF-I have been characterized in several other vertebrate species. The amino acid sequence of IGF-I is highly conserved among mammals. Daughaday and Rotwein (1989) demonstrated that 66 of 70 amino acid residues are identical among human, bovine, rat, and mouse IGF-I. Kajimoto and Rotwein (1989) isolated and characterized a IGF-I cDNA from the domestic chicken (*Gallus domesticus*) and reported a high correlation between the amino acid sequences of chicken and mammalian IGF-I. Similar observations have been reported for comparisons among the peptide sequences of mammalian IGF-I and those of an amphibian, *Xenopus laevis* (Shuldiner, et al. 1990) which actually appears to have two distinct IGF-I genes that represent two nonallelic IGF-I genes due to tetraploidy, and the coho salmon, *Oncorhynchus kisutch* (Cao et al., 1989). These studies indicate that the IGF-I gene has been well conserved throughout vertebrate evolution.

The protein product, however, remains to be identified in many of these non-mammalian species. Evidence for the presence of IGFs in non-mammalian vertebrates is scarce. IGF-I activity has been demonstrated in the serum of birds, reptiles, amphibians, and fish (Shapiro and Pimstone, 1977; Wilson and Hintz, 1982; Daughaday et al., 1985; Zangger et al., 1987; Funkenstein et al., 1989; Bautista et al., 1990; Crain, 1994). These data indicate the existence of some circulating forms of IGF-I, but little data exists concerning the sites of its production and storage. Reinecke et al. (1991) demonstrated the presence of IGF-I-like-immunoractivity in the brain, intestine, and pancreas of the Atlantic hagfish. Insulin-like growth factor-I-like immunoreactivity has also been detected in the oviduct of the vitellogenic alligator, *Alligator mississippiensis* (Cox and Guillette, 1993).

Drakenberg et al. (1993) reported the existence of IGF-I receptors in three lower vertebrates: *Cottus scorpius* (osteichthyes), *Raja clavata* (chondrichthyes), and *Myxine glutinosa* (agnatha). They identified the presence of IGF-I receptors in the brain of all three animals but did not detect the presence of IGF-II-mannose-6-phosphate receptors suggesting a biological role for an IGF-I-like molecule in lower vertebrates.

Biological actions

The IGFs are anabolic hormones. Their primary biological actions can be categorized as either acute insulin-like metabolic actions (rapid) or long-term growth-promoting actions (slow). Several lines of evidence elucidate the important role of IGF-I in growth. First, the pygmies living in central Africa are dwarfs, having normal pituitary GH secretion but are deficient in IGF-I (Merimee et al., 1981). The Laron dwarfs have very low plasma IGF-I concentrations in the presence of elevated plasma growth hormone levels (Zapf et al., 1984). A similar phenomenon is found in poodles. Eigenmann et

al. (1984) found that the toy poodle, which reaches a final weight between 2 and 3 kg, has extremely low IGF-I levels, which are approximately 10% of those found in the standard poodle. Second, experimental evidence has shown that IGF-I is capable of stimulating growth in hypophysectomized rats in the complete absence of growth hormone (Schoenle et al., 1982, 1985).

The IGFs elicit classical insulin effects on the typical target organs, especially adipose and muscle tissue. The IGFs increase glucose metabolism of adipose tissue (Froesch et al., 1966); however, they are much less potent in exerting metabolic effects on adipose tissue than insulin. Data support the hypothesis that the effects of the IGFs are elicited via the insulin receptor (Zapf et al., 1981). The IGFs stimulate the metabolism of striated muscle (Froesch et al., 1966; Oelz et al., 1970; Meuli and Froesch, 1975; Poggi et al., 1979), and their potent insulin-like action in muscle appears to be mediated through the IGF-I receptor.

The *in vivo* effects of the IGFs depend on their mode of administration. In rats, intravenous bolus injections of IGFs cause acute insulin-like effects on glucose homeostasis and metabolism, whereas long-term subcutaneous administration results in growth (Schoenle et al., 1982; Skottner et al., 1987; Guler et al., 1989). Bern et al. (1991) reported a similar phenomenon in the brook trout, *Salvelinus fontinalis*. This difference in response to bolus injection or constant infusion of IGFs has been hypothesized to be the result of a difference in the availability of the IGFs to target cells. Bolus injections result in an excess of free peptide (not bound to IGFBP) thus producing insulin-like effects. This hypothesis is supported by the finding that the truncated IGF-I that exhibits weak binding to the 25Kd IGFBP elicits a greater insulin-like metabolic response in rats as compared with intact IGF-I (Drakenberg et al., 1991)

The in vitro actions of the IGFs have been well established in a variety of cells, and these actions suggest an important role for these growth factors in cellular proliferation and differentiation. The IGFs stimulate primary chick embryo fibroblast cultures (Zapf et al., 1978). Mammalian fibroblast cell lines are also stimulated to synthesize DNA and to replicate by IGF-I and IGF-II (Froesch et al., 1979). It is interesting to note that pure IGFs alone or in combination are not adequate substitutes for whole serum, even in large quantities, suggesting the interaction of other growth factors in the process of cellular proliferation and differentiation. The IGFs have also been shown to stimulate the differentiation of neural tissue (McMorris et al., 1986), myoblasts (Ewton and Florini, 1981; Schmid et al., 1983), osteoblasts (Schmid et al., 1984), and adipocytes (Ewton and Florini, 1981; Smith et al., 1988). The IGFs appear to play a role in differentiation in both the ovary and the testis (Chatelain et al., 1987; Veldhuis et al., 1986; Giudice, 1992).

In birds, IGF-I has been shown to be important in embryonic development (Serrano et al., 1989) and in the promotion of fiber cell elongation in lens cells (De Pablo et al., 1990). IGF-I stimulates transepithelial sodium transport in the urinary bladder of the toad *Bufo marinus*, a model renal epithelium (Blazer-Yost et al., 1992). Growth hormone stimulates hepatic and extrahepatic production of IGF-I which in turn mediates many of the growth-promoting activities of growth hormone in the teleost *Gillichthys mirablis* (Gray and Kelley, 1991). Cao et al. (1989) demonstrated a sixfold increase in prepro-IGF-I mRNA in liver RNA isolated from salmon injected with bovine growth hormone. Perez-Sanchez et al. (1992) showed that IGF-I inhibits growth hormone release from cultured rainbow trout pituitary cells. According to the results of one study, human IGF-I does not stimulate growth in the brook trout, *Salvelinus fontinalis*. Instead, high doses cause

profound insulin-like effects resulting in hypoglycemia (Skyrud et al., 1988). Bern et al. (1991) reported similar results when bolus injections of hIGF-I were given to brook trout. However, when the animals received a constant infusion of recombinant bovine IGF-I, stimulation of growth occurred.

The generalized cell cycle is divided into 3 distinct phase: G_1 (Gap 1), S (DNA synthesis phase), and G_2 (Gap 2). Cells not undergoing cellular division exist in the quiescent G_0 phase. A major control site for the initiation of cellular division is in the transition from the G_0 to the G_1 phase. It has been suggested that the IGFs act as progression factors to stimulate cells through the DNA synthesis phase of the cell cycle. Other growth factors, such as FGF and PDGF, serve as competence factors which initiate the cell cycle and prime the cells to respond to progression factors (Stiles et al., 1979). Van Wyk et al., (1984) demonstrated that various growth factors had definite roles during the cell cycle as cells moved from the quiescent G_0 state to the G_1 phase and through DNA synthesis in the S phase. Using mouse Balb-c/3T3 fibroblasts, basic FGF and PDGF were shown to act as competence factors, enabling cells to pass from the G_0 to the G_1 stage of the cell cycle but had no further role at later points in the cycle. EGF, on the other hand, played a role during the first part of the G_1 phase, and either IGF-I, IGF-II, or insulin was necessary for the final part of G_1 and for entry into the S phase. When present together, these growth factors appear to act synergistically to regulate cellular proliferation. These growth factors also play a role during cellular differentiation. For example, IGF potentiates the differentiation of myoblast cell lines, such as rat L6 cells, into postmitotic contractile myotubes. This process which occurs spontaneously as myoblasts grow to high cell density, can be stimulated to occur prematurely with IGF-I. The initial effects of IGF-I include at least one round of cell proliferation prior to terminal

differentiation and activation of specific genes such as myogenin. Basic FGF also induced myoblast proliferation but inhibited terminal differentiation (Linkhart et al., 1981). TGF-β also prevented terminal differentiation and had little effect on proliferation. There are a multitude of growth factors in the cellular microenvironment. Exactly how precise cellular specificity can be attained by ubiquitously expressed growth factors remains to be determined

Research Objectives

A summary diagram of potential roles for IGF-I and EGF in reptilian reproduction based on the mammalian model is displayed in Figure 1-2. Under stimulation of GnRH released from the hypothalamus, the anterior pituitary gland releases FSH and LH. These gonadotropins act on the ovary to stimulate follicular growth and steroidogenesis. The steroid hormones E_2 and P_4 stimulate growth and differentiation of the oviduct. Evidence is accumulating that the steroid-induced oviductal response is mediated by polypeptide growth factors such as IGF-I and EGF. In addition to mediating the effects E_2 and P_4-induced oviductal growth and differentiation, IGF-I is a uterine secretory product in mammals and is involved in early embryonic development. In reptiles, IGF-I could be incorporated into the egg as it passes along the oviduct prior to oviposition. Our current understanding of polypeptide growth factors associated with mammalian reproduction has led to the formation of three major hypotheses. First, I hypothesize that the polypeptide growth factors IGF-I and EGF are present in the reptilian oviduct and that they are involved in mediating estrogen-induced uterine proliferation and differentiation. My second hypothesis is that immunostaining patterns for these growth factors will vary throughout the

reproductive cycle. Third, I hypothesize that IGF-I is a component of the reptilian egg and is involved in early embryonic development.

In an attempt to test these hypotheses, this dissertation will address the following research questions: (1) Are the polypeptide growth factors, EGF and IGF-I, present in the reptilian oviduct? IGF-I-like immunoreactivity has been detected in reptiles, but few data exist concerning the presence of either IGF-I or EGF in the reproductive tract. Insulin-like growth factor-I-like immunoreactivity has been detected in the vitellogenic oviduct of a single reptilian species, the American alligator, *A. mississippiensis* (Cox and Guillette, 1993). (2) If present, does EGF and IGF-I immunoreactivity vary with oviductal region and with reproductive condition in the American alligator, *A. mississippiensis*? In mammals, these growth factors have been shown to be regulated in the reproductive tract by many hormones including E_2. One of the goals of this study is to determine whether changes in patterns of immunocytochemical staining for IGF-I and EGF correlate with fluctuating hormone levels during the reproductive cycle. (3) Do plasma IGF-I concentrations vary with reproductive condition in the alligator? (4) Is IGF-I synthesized by the alligator oviduct and are IGF-I mRNA levels in the oviduct related to circulating steroid hormone levels? (5) Is IGF-I present in the albumen and yolk of alligator eggs and if present, do IGF-I levels change during embryonic development? (6) Do EGF and IGF-I function as mediators of estrogen-induced oviductal growth in the reproductive tract of the Mediterranean gecko, *Hemidactylus turcicus*?

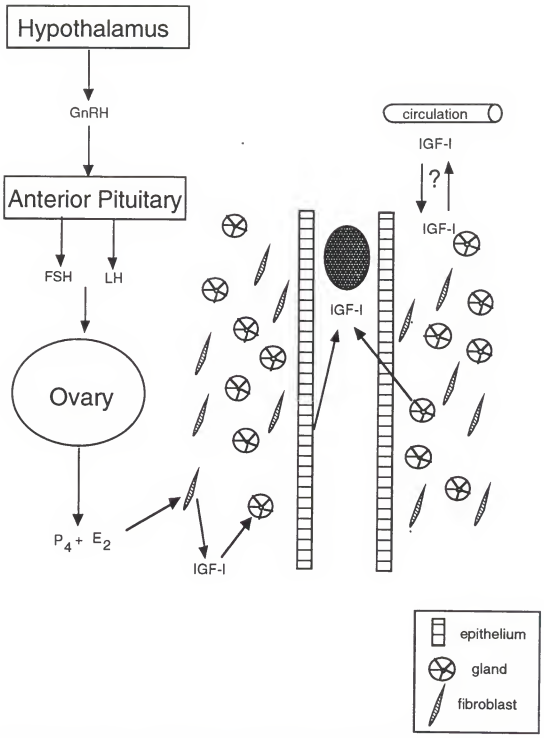

Figure 1-2. Summary diagram of potential roles of IGF-I in reptilian reproduction based on the mammalian model.

CHAPTER 2
IMMUNOCYTOCHEMICAL LOCALIZATION OF INSULIN-LIKE GROWTH FACTOR I AND EPIDERMAL GROWTH FACTOR IN THE REPTILIAN OVIDUCT

Introduction

The primitive amphibians gave rise to the stem reptiles (cotylosaurs) which diverged into several evolutionary lineages of which only four have extant representatives (Fig. 2-1). The subclass Anapsida diverged early in reptilian evolution and gave rise to the order Chelonia (turtles, tortoises, and terrapins). A second lineage, the subclass Lepidosaura, gave rise to two extant reptilian groups, the order Squamata (lizards and snakes) and the order Sphenodontia (tuatara). The subclass Archosaura represents the third lineage of reptiles which gave rise to the order Crocodilia (crocodiles and alligators) and class Aves (birds). A final pathway, subclass Synapsida, separated very early from the main reptilian line and gave rise to the mammals.

Oviductal anatomy and physiology vary significantly among the reptilian orders. The tuatara (*Sphenodon punctatus*) possesses the simplest and most generalized functional reproductive tract of the extant reptiles, whereas the alligator (*Alligator mississippiensis*) has the most specialized reproductive tract. The reptilian oviduct is divided into four functionally distinct regions: infundibulum, tube, uterus, and vagina. Differences in oviductal morphology provide the basis for the distinctive modes of eggshell formation seen in reptiles. In all reptiles studied, the tube region is the site of albumen production, whereas the uterus is responsible for formation of the

eggshell layers (fibers, calcium coat). In the tuatara, both layers of the eggshell, the fibrous layer and the calcareous layer, are produced by the uterus simultaneously. In turtles and squamates, both layers are produced by a single uterine region, but there is a temporal separation of membrane formation and calcareous shell secretion. Crocodilians are different from all other reptiles but similar to their archosaurian relatives, the birds, in having a uterus which is divided into an anterior fiber-secreting region and a posterior calcium-secreting region. The tube region of the alligator oviduct is similar in functional morphology to the avian magnum, whereas the fiber uterus and calcium uterus are homologous to the avian isthmus and shell gland respectively.

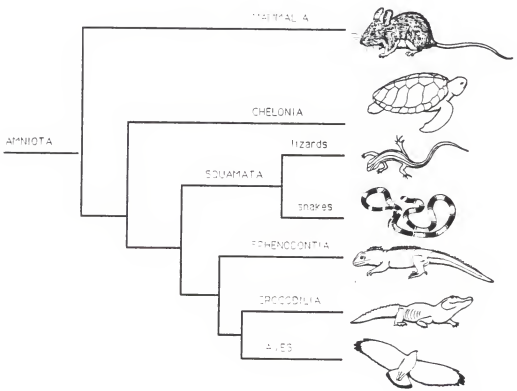

Figure 2-1. Phylogenetic relationships among extant reptilian groups.

Hormonal regulation of the seasonal development of the reptilian oviduct, as in other vertebrates, involves the steroid hormones estradiol-17β and progesterone. During follicular development in the alligator, ovarian follicles increase in size and secrete estradiol in response to pituitary gonadotropin secretion (Lance, 1989). Estradiol secreted by the developing follicles serves two basic functions in reptiles. First, estradiol stimulates the liver to synthesize the yolk precursor protein vitellogenin which is then secreted into the blood, carried to the ovaries, taken up by the oocytes, and transformed into yolk (Ho, 1982). Second, estradiol stimulates growth and differentiation of the reproductive tract in preparation for oviposition (Mead et al., 1981; Jones, 1982; Guillette et al., 1991). Estrogen stimulation of oviductal growth in alligators (Forbes, 1938), iguanid lizards (Callard, 1972) and snakes (Mead et l., 1981) indicates that estrogens serve a similar function in all reptiles.

One concept which has emerged during the past decade is that estrogens might stimulate cellular proliferation and differentiation indirectly through mitogenic polypeptides. Both IGF-I (Murphy and Friesen, 1989) and EGF (DiAugustine et al., 1988) have been implicated as possible mediators of estrogen action in the mammalian uterus. IGF-I has been identified in reptiles (Fig. 2-2), but its influence on reptilian reproduction has not been studied. The purposes of this study was to determine if IGF-I and EGF are present in the oviduct of representative reptiles and, if present, whether or not patterns of immunoreactivity vary among oviductal regions.

Table 2-1. Insulin-like growth factor I in reptiles.

Species	Technique	Reference
P. scripta elegans	RIA	Daughaday et al., 1985
Lizard-gecko	RIA	Bautista et al., 1990
S. virgatus	ICC	Cox & Matter, Unpublished
G. polyphemus	ICC	Cox, Unpublished
A. misssissippiensis	ICC	Cox & Guillette, 1993
A. misssissippiensis	RIA	Cox , Unpublished
T. scripta elegans	RIA	Crain, 1994
C. caretta	RIA	Crain, 1994

Materials and Methods

Specimens

Tuatara (*S. punctatus*), gopher tortoise (*Gopherus polyphemus*), lizard (*Sceloporus virgatus*), and American alligator (*A. mississippiensis*) oviducts were examined in this study. Tuatara tissue from three oviductal regions (tube, anterior uterus, and posterior uterus) was provided by Dr. A. Cree, University of Otago, Dunedin, New Zealand. The oviducts of five vitellogenic gopher tortoises were obtained from the collection of the Florida Museum of Natural History. These specimens were collected from Alachua, Putnam, and Marion counties, Florida, killed by ethanol injection into the brain within 48 hours of capture (Taylor, 1982) and have been used for anatomical studies of the reproductive tract by several authors (Taylor, 1982; Palmer, 1987; Palmer and Guillette, 1988; Palmer, 1990). Five lizards (*S. virgatus*) which were captured in Cochise County, Arizona were euthanized and their oviducts were fixed in Bouin's fixative for 24 hours and transferred to 75% ethanol. Alligator oviducts were obtained from 5 mature, vitellogenic

animals which were captured from 3 different lakes (Griffin, Orange, and Okeechobee) in central Florida (GFC permit #W88063). Within 24 hours of capture, the alligators were anaesthetized with 20 mg/kg sodium pentobarbital, and their oviducts were surgically removed. Tissue from the 3 major functional regions of the oviduct (tube, fiber-secreting uterus, and calcium-secreting uterus) were dissected and immediately fixed in Bouin's fixative for 3-7 days and transferred to 75% ethanol.

Basic Histology

Tissues were washed in 75% ethanol, dehydrated in a series of graded ethanols, cleared in xylene, embedded in paraffin, and serially sectioned at 8μm on a rotary microtome and mounted on slides (Humason, 1981). Five slides for each species were stained with a connective tissue staining technique using hematoxylin, eosin, Alcian blue (pH 2.5 for glycosaminoglycans), fast green (for connective tissue), orange G (for red blood cells), and Biebrich scarlet (for proteins) dyes. The remainder of the slides were used for immunocytochemical validation and experimentation.

Immunocytochemistry

Validation of the immunocytochemical procedure was accomplished with (1) negative controls: treating control slides without primary antibody or with nonimmune normal rabbit serum purchased from Sigma Chem. Co., St. Louis, MO (similar results were obtained from these two control procedures) for each experimental slide, and (2) positive controls: exposing pig oviductal tissue known to be positive for both IGF-I and EGF as positive controls for each individual immunocytochemisty experiment. Additionally, initial validation of the procedure included saturating the primary antibodies (anti-

IGF-I and anti-EGF) with their respective antigens (IGF-I and EGF) prior to incubation of the tissue with primary antibody. Three different commercial immunostaining techniques were used: two immunoperoxidase staining kits, Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA and Histostain-SP Kit, Zymed Laboratories, South San Francisco, CA and an immunogold silverstaining procedure, intenSE M, Janssen Biotech, Olen, Belgium.

The anti-human IGF-I antibody (polyclonal anti-IGF-I/somatomedin C rabbit antiserum - Lot# UB3-189) was received as a gift from Drs. Louis Underwood and Judson J. Van Wyk, Division of Pediatric Endocrinology, University of North Carolina at Chapel Hill (distributed for research use by the Hormone Distribution Program of the National Institute of Diabetes and Digestive and Kidney Diseases through the National Hormone and Pituitary Program). The EGF antibody was purchased from Sigma Chem. Co., St. Louis, MO. Both of the primary antibodies were used at a final dilution of 1:1000 (10µl stock 1:10 antibody + 990µl tris buffered saline (TBS, pH 7.4). For the ABC kit, tissue sections were deparaffinized in xylene and hydrated in a series of graded ethanols (100%, 100%, 95%, 70%). Slides were then treated with 3% H_2O_2 for 30 minutes to block endogenous peroxidase activity, washed with TBS, and incubated with primary antibody diluted in 1.5% normal goat blocking serum (experimental sections) or without primary antibody (control sections). Tissues were then washed with TBS for 10 minutes, incubated with biotinylated secondary antibody for 10 minutes, and washed again with TBS for 10 minutes. Sections were incubated with Vectastain Elite ABC reagent for 30 minutes, washed with TBS for 10 minutes, and incubated with a peroxidase substrate solution (0.1% diaminobenzidene tetrachloride made in 0.1M tris buffered saline, pH 7.2 and mixed with equal amounts of 0.02%

H_2O_2) for 5 minutes to localize the bound primary antibodies. Finally, slides were washed in tap water and coverslips were mounted.

For the Histostain-SP Kit, slides were deparaffinized with xylene and rehydrated in a series of graded ethanols and place in a TBS (pH 7.5) bath for 10 minutes. Next, slides were incubated in a peroxidase quenching solution 3% H_2O_2 in methanol for 10 minutes and treated with a serum blocking solution for 10 minutes. Sections were then incubated with 100µl of primary antibody for 45 minutes and rinsed with TBS and incubated with 100µl of biotinylated secondary antibody and rinsed with TBS. Sections were treated with 100µl of enzyme conjugate and the presence of the enzyme was revealed by incubation of the tissue with the substrate-chromogen solution. Finally, slides were rinsed in dH_2O and mounted with coverslips.

Results

Validation

Control sections incubated without primary antibodies or with nonimmune normal rabbit serum exhibited minimal background or no immunostaining. Similarly, control sections incubated with primary antibodies that were preabsorbed with either human recombinant IGF-I or EGF exhibited a significant reduction (~75%) in immunoreactivity. Pig sections served as a positive control and stained positively for both IGF-I and EGF. Similar immunostaining patterns were observed with all 3 immunocytochemical procedures, thus I will report the observations, using all three techniques, as one data set.

Gopherus polyphemus

In the tube (Fig. 2-2), low-intensity IGF-I immunoreactivity was observed throughout the cytoplasm of all epithelial cells with more intense staining at the tips of these cells. Immunoreactive intensity varied in the stroma from a complete lack of staining to moderate staining. Low to moderate staining intensity was observed in the tubal glands with low-intensity immunoreactivity in the myometrium. In the uterus (Fig. 2-4), low -intensity inmmunoreactivity was observed throughout the cytoplasm of the epithelial cells with moderate to high-intensity immunoreactivity at the apical tips. High-intensity immunoreactivity was observed in the endometrial glands whereas the myometrium exhibited low to moderate-intensity.

The epithelial cells of the tube (Fig. 2-2) showed low-intensity immunoreactivity for EGF with moderate-intensity staining occurring at the apical tips of some cells. Low-intensity immunoreactivity for EGF was also observed in the stroma, endometrial glands, and myometrium of the tube. Low-intensity immunostaining was seen in the uterine epithelial cells (Fig. 2-3) with low to moderate-intensity staining at the apical tips. Low-intensity staining seen in the stroma, moderate intensity in the glands, and low to moderate intensity in the myometrium was observed.

Sceloporus virgatus

The tube and uterus obtained from vitellogenic female *S. virgatus* were studied. In the tube (Fig. 2-4), IGF-I immunoreactivity was observed in the apical tips of epithelial cells, cytoplasm of the epithelial cells, tubal glands,

stroma, and myometrium. The apical tips of the epithelial cells consistently stained more intensely than the other four regions. Interestingly, the intense staining at the apical tips of the epithelial cells appeared to be associated with the ciliated epithelial cells more often than the non-ciliated secretory cells. Blood cells showed intense immunoreactivity in experimental but not control sections. In the uterus (Fig. 2-5), intense immunoreactivity was noted in the apical tips of the epithelial cells with slightly less intense staining throughout the cytoplasm of the epithelial cells. Intense staining was also observed in the endometrial glands. The stroma and the myometrium exhibited moderately-intense staining.

EGF immunostaining patterns were less consistent than those observed for IGF-I. In two of the oviducts examined, staining in the tube region was not observed in either the apical tips or the cytoplasm of the epithelial cells. In the other three oviducts, immunostaining was observed in both of these areas with very intense staining in the apical tips of the epithelial cells (Fig. 2-4). The glands stained intensely in one animal and moderately in the other three animals. Both the stroma and the myometrium exhibited moderate staining in all five animals. In the uterus (Fig. 2-5), a similar pattern was observed. No immunoreactivity was observed in the epithelial cells of the two animals studied, whereas it was observed in both the apical tips and throughout the cytoplasm of the epithelial cells of the other three animals. Staining was observed in the endometrial glands of all the uteri observed. Moderate staining was observed in the stroma and myometrium.

Alligator mississippiensis

IGF-I immunoreactivity exhibited similar patterns in the tube, fiber uterus, and calcium uterus. Immunostaining for IGF-I in the tube (Fig. 2-6) was observed within the cells comprising the glands and in the myometrium. Intense staining at the apical aspect of the epithelial cells was also noted. Little or no staining occurred throughout the cytoplasm of the epithelial cells, in the glands, or in the stroma. Immunostaining results from the fiber uterus (Fig. 2-7) were difficult to interpret due to high levels of background (nonspecific) staining. This high background staining may have resulted from either high levels of endogenous peroxidase activity or high levels of avidin or biotin within this oviductal region. Nonspecific staining was most prevalent in the glands. Immunostaining in the calcium-secreting region of the uterus (Fig. 2-8) also occurred in the epithelial cells, glands, and myometrium with little or no staining in the stroma.

EGF immunoreactive patterns were different when compared to IGF-I staining. The most immunoreactive area of the tube was the tips of the epithelial cells (Fig. 2-6). Staining also occurred throughout the cytoplasm of the epithelial cells, in the glands, and in the myometrium, but immunoreactivity was absent in the stroma. Minimal staining occurred in the epithelial cells and in the myometrium of the fiber uterus. No staining was observed in the stroma of this region. Again, as described above, immunostaining results in the glands of the fiber region (Fig. 2-7) were inconclusive due to high background staining. Interestingly, EGF immunoreactivity was absent in the calcium region of all three animals which were classified as early-vitellogenic whereas the two animals classified

as mid to late vitellogenic exhibited immunoreactivity in epithelial cells, glands, stroma, and myometrium (Fig. 2-8).

Sphenodon punctatus

Two regions of the oviduct (tube and uterus) of a single vitellogenic animal were examined. In the tube, IGF-I immunoreactivity was observed in the apical tips of the epithelial cells but not throughout the cytoplasm of the cells. Low-intensity staining was observed in the stroma, whereas high-intensity staining was observed in the myometrium. No staining was seen in the oviductal glands. High intensity immunoreactivity was observed in the red blood cells filling the capillaries in the myometrium. No EGF immunoreactivity was observed in the tube.

In the uterus (Fig. 2-9), low-intensity immunoreactivity was observed in the epithelial cells (at the tips and throughout the cells). Endometrial glands exhibited differential staining patterns. Some glands, exhibit no immunoreactivity, whereas other oviductal glands exhibited low to moderate levels of immunoreactivity. Low-intensity staining was also present in both the stroma and the myometrium. Low-intensity immunostaining for EGF was observed only in the uterine glands.

Discussion

IGF-I-like and EGF-like immunoreactivity is present in the oviducts of the tuatara (*S. punctatus*), a gopher tortoise (*G. polyphemus*), a lizard (*S. virgatus*), and alligator (*A. mississippiensis*). Specifically, immunoreactivity was observed in all regions of the oviductal tissue: apical tips of the epithelial cells, throughout the cytoplasm of the epithelial cells, in the glands, throughout the stroma, and in the myometrium. These data are similar to

that reported in the mammalian literature. Ghahary et al. (1990) reported the presence of IGF-I in the luminal epithelia, in the outer longitudinal and inner circular layers of the myometrium, and in the stroma of rat uteri using in situ hybridization. Positive immunostaining for IGF-I-like and EGF-like material in the reptilian oviduct using heterologous polyclonal antibodies suggests a high degree of homology between the reptilian forms of these growth factors and their mammalian counterparts as reported for IGF-I in chicken, frog, and salmon (Cao, et al., 1989; Kajimoto and Rotwein, 1989; Shuldiner, et al., 1990). Furthermore, data which demonstrate growth-hormone regulated expression of IGF-I in coho salmon, *Oncorhynchus kisutch*, (Cao, et al., 1989) domestic chicken, *Gallus domesticus*, (Kajimoto and Rotwein, 1989), and in several mammals (Murphy and Friesen, 1988) indicate that the conserved primary structure and growth hormone-regulated expression of IGF-I may occur throughout the vertebrates.

Chan et al. (1990) cloned a hybrid insulin/insulin-like growth factor cDNA from amphioxus (*Branchiostoma californiensis*), a primitive cephalochordate that occupies a key position in chordate phylogeny which may represent a transitional form connecting insulin and IGF. IGF probably arose at an early stage in vertebrate evolution from an ancestral insulin gene. This high degree of conservation of structure and hormone-regulated expression supports a fundamental role for IGF-I in general aspects of growth, development, and differentiation.

The presence of IGF-I and EGF in the oviduct of vitellogenic reptiles suggests a role for these growth factors in reptilian reproduction. These growth factors could stimulate oviductal growth and differentiation during the reproductive cycle. Mead et al. (1981) demonstrated the important role of E_2 in uterine development in a garter snake *Thamnophis elegans*, but also

reported that additional hormones may be required to fully restore the uterus to preovulatory condition since administration of E_2 alone to ovariectomized snakes failed to completely restore the uterus to such a state. The many *in vitro* actions of the IGFs which have been well established in a variety of cell types (see Chapter 1) suggest an important role for growth factors in cellular proliferation and differentiation. The presence of IGF-I and EGF in the reptilian oviduct during vitellogenesis when circulating E_2 levels are elevated and the reproductive tract is undergoing growth and development in preparation for egg laying supports the hypothesis that these growth factors are involved in oviductal proliferation and differentiation.

Both IGF-I and EGF have been implicated as possible mediators of E_2 action in the mammalian oviduct (Murphy et al, 1987; DiAugustine et al., 1988). The presence of these growth factors in the vitellogenic reptilian oviduct when circulating E_2 levels are elevated suggests a similar role for IGF-I and EGF in reptiles. In addition to serving as local mediators of E_2, IGF-I and EGF might also be components of the egg and play an important role in regulation of embryonic development in reptiles.

Figure 2-2. Immunocytochemical localization of IGF-I and EGF in the oviductal tube of a vitellogenic gopher tortoise *G. polyphemus* (400X) A: Basic histology: epithelium (e), gland (g), stroma (s), and myometrium (m). B: Experimental tissue incubated with polyclonal anti-human IGF-I rabbit antiserum. C: Experimental tissue incubated with polyclonal anti-mouse EGF rabbit antiserum. D: Control tissue incubated with non-immune normal rabbit serum.

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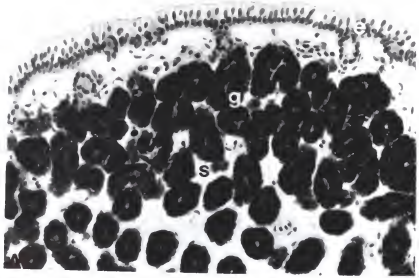
Figure 2-3. Immunocytochemical localization of IGF-I and EGF in the uterus of a vitellogenic gopher tortoise *G. polyphemus*. (200X)A: Basic histology: epithelium (e), gland (g), stroma (s), and myometrium (m). B: Experimental tissue incubated with polyclonal anti-human IGF-I rabbit antiserum. C: Experimental tissue incubated with polyclonal anti-mouse EGF rabbit antiserum. D: Control tissue incubated with non-immune normal rabbit serum.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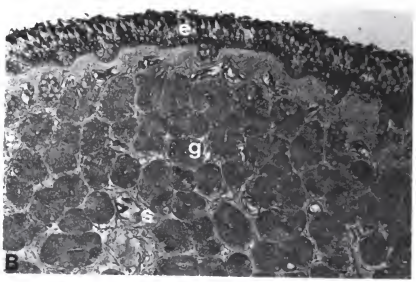
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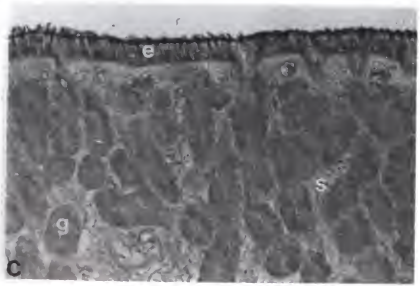
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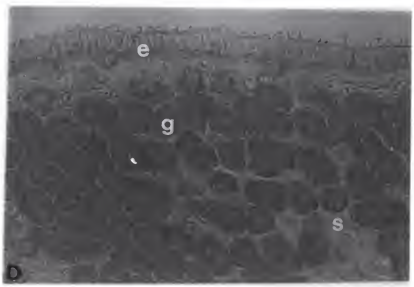
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Figure 2-4. Immunocytochemical localization of IGF-I and EGF in the oviductal tube of a vitellogenic lizard *S. virgatus* (400X) A: Experimental tissue incubated with polyclonal anti-human IGF-I rabbit antiserum. epithelium (e), gland (g), stroma (s), and myometrium (m). B: Experimental tissue incubated with polyclonal anti-mouse EGF rabbit antiserum. C: Control tissue incubated with non-immune normal rabbit serum.

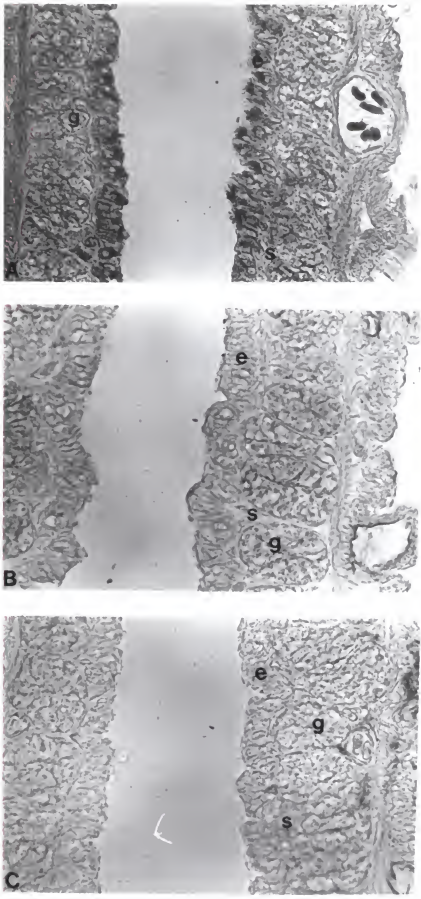
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Figure 2-5. Immunocytochemical localization of IGF-I and EGF in the uterus of a vitellogenic lizard *S. virgatus* (400X) A: Basic histology: epithelium (e), gland (g), stroma (s), and myometrium (m). B: Experimental tissue incubated with polyclonal anti-human IGF-I rabbit antiserum. C: Experimental tissue incubated with polyclonal anti-mouse EGF rabbit antiserum. D: Control tissue incubated with non-immune normal rabbit serum.

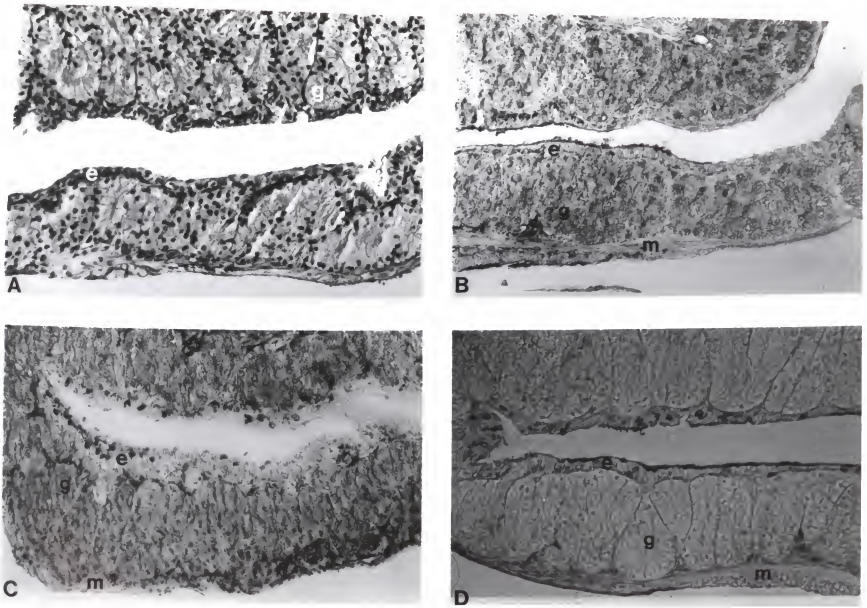
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Figure 2-6. Immunocytochemical localization of IGF-I and EGF in the oviductal tube of a vitellogenic American alligator *A. mississippiensis* (200X)A: Basic histology: epithelium (e), gland (g), stroma (s), and myometrium (m). B: Experimental tissue incubated with polyclonal anti-human IGF-I rabbit antiserum. C: Experimental tissue incubated with polyclonal anti-mouse EGF rabbit antiserum. D: Control tissue incubated with non- immune normal rabbit serum.

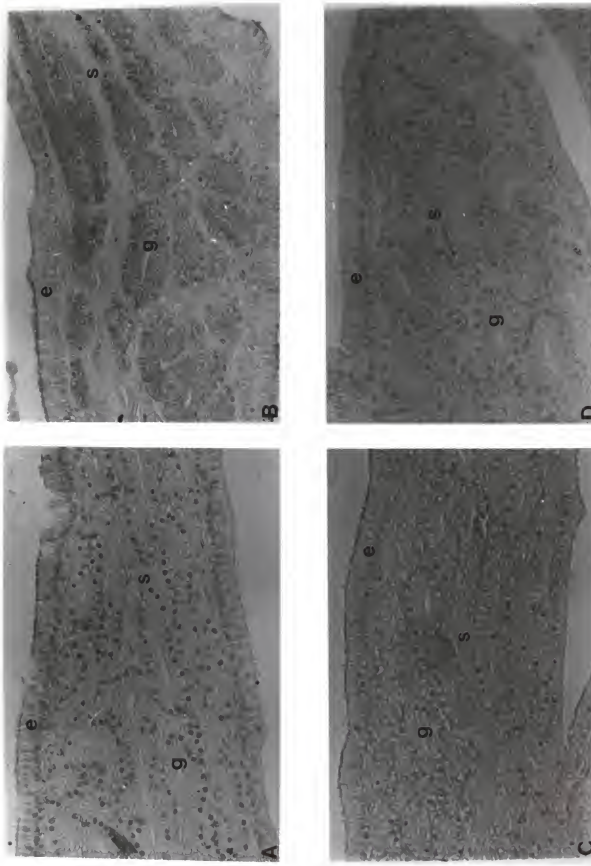
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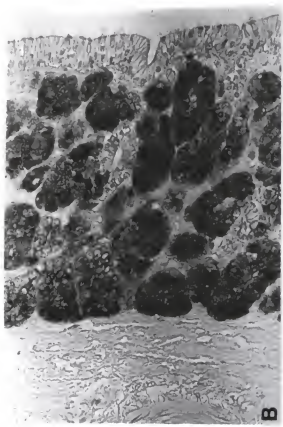
Figure 2-7. Immunocytochemical localization of IGF-I and EGF in the fiber uterus of a vitellogenic American alligator, *A. mississippiensis* (200X)A: Basic histology: epithelium (e), gland (g), stroma (s), and myometrium (m). B: Experimental tissue incubated with polyclonal anti-human IGF-I rabbit antiserum. C: Experimental tissue incubated with polyclonal anti-mouse EGF rabbit antiserum. D: Control tissue incubated with non-immune normal rabbit serum.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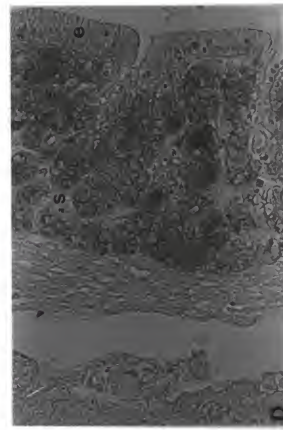
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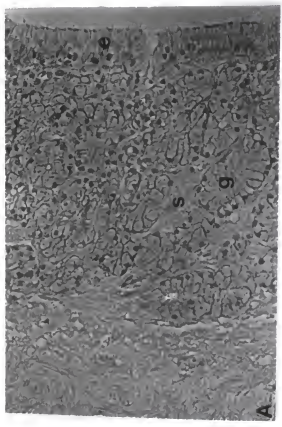
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Figure 2-8. Immunocytochemical localization of IGF-I and EGF in the calcium uterus of a vitellogenic American alligator *A. mississippiensis* (200X)A: Basic histology: epithelium (e), gland (g), stroma (s), and myometrium (m). B: Experimental tissue incubated with polyclonal anti-human IGF-I rabbit antiserum. C: Experimental tissue incubated with polyclonal anti-mouse EGF rabbit antiserum. D: Control tissue incubated with non- immune normal rabbit serum.

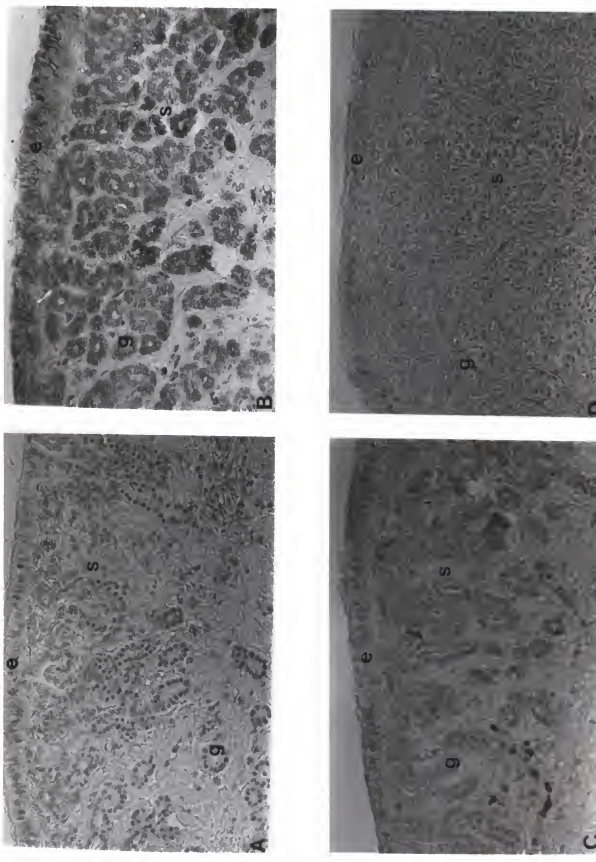
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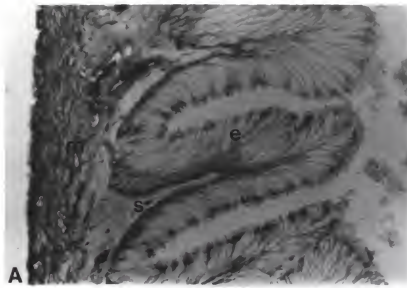
Figure 2-9. Immunocytochemical localization of IGF-I and EGF in the uterus of a vitellogenic tuatara *S. punctatus* (200X) A: Basic histology: epithelium (e), stroma (s), and myometrium m). B: Experimental tissue incubated with polyclonal anti-human IGF-I rabbit antiserum. C: Experimental tissue incubated with polyclonal anti-mouse EGF rabbit antiserum. D: Control tissue incubated with non-immune normal rabbit serum.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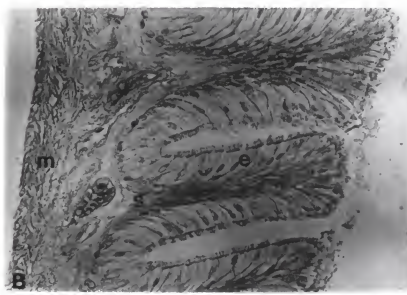
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CHAPTER 3
IMMUNOCYTOCHEMICAL LOCALIZATION OF INSULIN-LIKE GROWTH FACTOR I AND EPIDERMAL GROWTH FACTOR IN THE OVIDUCT OF THE AMERICAN ALLIGATOR, *ALLIGATOR MISSISSIPPIENSIS*

INTRODUCTION

The functional morphology of the oviduct of the American alligator, *A. mississippiensis*, differs from that described for other reptiles (Palmer and Guillette, 1992). Although neonatal alligators exhibit no regional specialization of the oviduct (Austin, 1990), sexually mature females (those greater than 1.8 m in length) have an oviduct differentiated into five structurally and functionally distinct regions: an infundibulum which receives eggs following ovulation, a tube region that secretes albumen, a uterus which is divided into an anterior, fiber-secreting region and a posterior, calcium-secreting region, and a vagina (Palmer and Guillette, 1992). The tube region of the alligator oviduct is similar in functional morphology to the avian magnum, whereas the fiber-secreting region of the uterus and the calcium-secreting region of the uterus are homologous to the avian isthmus and shell gland, respectively. Growth factors in the reptilian oviduct might be involved in at least two very important reproductive functions: seasonal development of the reproductive tract and embryonic development. Seasonal proliferation and differentiation of the oviduct is controlled by estrogens, primarily estradiol-17β (E_2). However, E_2 action appears to be mediated by polypeptide growth factors such as insulin-like growth factor

(IGF-I) (Murphy and Friesen, 1988), epidermal growth factor (EGF) (Nelson et al., 1990), platelet derived growth factor (PDGF) (Ronnstrand et al., 1987) and transforming growth factor-α (TGFα) (Nelson et al., 1992). Although the tube of the alligator is similar in morphology to that of a bird, the proteins secreted from this region are unique when compared to birds (Palmer, 1990; Palmer and Guillette, 1992). In addition to unique egg white proteins, alligator albumen also contains IGF-I (Guillette and Williams, 1991; Chapter 6). The role of IGF-I in the albumen is unknown, although this hormone has been implicated in the control of mammalian embryonic development (Simmen and Simmen, 1990). Insulin-like growth factor-I and epidermal growth factor are present in the reptilian oviduct (Chapter 2) and are likely candidates for such roles. The purpose of this study is to determine whether IGF-I and EGF immunoreactivity varies with oviductal region and reproductive condition in the American alligator *A. mississippiensis*.

Materials and Methods

Specimens

Twenty-seven mature, female alligators (*A. mississippiensis*) were captured from several lakes (Griffin, Orange, Okeechobee) in central Florida (Permit #W88063). Within 24 hours of capture, the animals were anesthetized with 20 mg/kg sodium pentobarbital, and their oviducts were surgically removed. Animals were categorized based on gross reproductive state and/or date of capture as either non-reproductive (n=9), early-vitellogenic (n=3), mid-vitellogenic (n=3), late vitellogenic (n=3), early-gravid (n=3), mid-gravid (n=3), late gravid (n=3), early-postoviposition (n=3), mid-postoviposition (n=3), or late postoviposition (n=3). Tissues from the three

major functional regions of the reproductive tract (tube, fiber-secreting uterus, and calcium-secreting uterus) were dissected and immediately fixed for basic histology and immunocytochemistry in Bouin's fixative (see Palmer and Guillette, 1992, for details of oviductal morphology). These animals were taken as part of a larger interdisciplinary research project (University of Florida, U.S. Fish and Wildlife, Florida Game and Freshwater Fish Commission) on the reproductive biology of the alligator.

Basic Histology

Tissues were washed, dehydrated in a series of graded ethanols, cleared in xylene, embedded in paraffin, and serially sectioned at 8 µm on a rotary microtome (Humason, 1981). Sections from each oviductal region of each alligator (tube, fiber uterus, calcium uterus) were gelatin-mounted on slides. Each slide had one section from each oviductal region of a single animal. Fifteen slides were prepared for each animal. Each set of fifteen slides contained adjacent sections for each oviductal region so that comparisons of individual cells and glands could be made. Two slides from each set were stained with a modified Shorr's connective tissue staining technique using hematoxylin (nuclear stain), eosin (counterstain), Alcian blue (pH 2.5 for glycosaminoglycans), fast green (for connective tissue), orange G (for red blood cells), and Biebrich scarlet (for proteins). The remainder of the slides were used for immunocytochemical validation and experimentation.

Immunocytochemical Staining

Validation

Validation of the immunocytochemical procedure was accomplished by (a) treating control slides without primary antibody or with non-immune

normal rabbit serum (similar results were obtained from these two control procedures) for each experimental slide incubated with primary antibody, and (b) exposing pig oviductal tissue known to be positive for IGF-I and EGF as a control with each set of gator slides.

Experimentation

The anti-human IGF-I antibody (polyclonal anti-IGF-I/somatomedin C rabbit antiserum - Lot #UB3-189) was received as a gift from Drs. Louis Underwood and Judson J. Van Wyk, Division of Pediatric Endocrinology, University of North Carolina at Chapel Hill (distributed for research use by the Hormone Distribution Program of the National Institute of Diabetes and Digestive and Kidney Diseases through the National Hormone and Pituitary Program). The EGF antibody was purchased from Sigma Chem. Co. (St Louis, MO). Both of the antibodies were used at a final dilution of 1:1000 (10 µl stock 1:10 antibody + 990µl, TBS pH 7.4). Immunocytochemical staining was performed using one of two immunoperoxidase systems, Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA) or Histostain-SP Kit (Zymed Laboratories, South San Francisco, CA). Tissue sections were deparaffinized in xylene and hydrated in graded alcohols.

For the Vectastain protocol, slides were treated with 3.0% H_2O_2 for 30 minutes to block endogenous peroxidase activity, washed with tris buffered saline (TBS, pH 7.4), and incubated with primary antibody diluted in 1.5% normal goat blocking serum (experimental sections) or without primary antibody (control sections). Tissues were then washed with TBS (pH 7.4) for 10 minutes, incubated with biotinylated secondary antibody, and washed with TBS (pH 7.4) for 10 minutes. Sections were then incubated with Vectastain Elite ABC reagent for 30 minutes, washed with TBS (pH 7.4) for 10 minutes, and incubated with a peroxidase substrate solution (0.1% diaminobenzidine

tetrachloride made in 0.1M tris buffer, pH 7.2 and mixed with equal amounts of 0.02% H_2O_2) for 5 minutes to localize the bound primary antibodies. Finally, slides were washed in tap water and coverslips were mounted.

For the Histostain-SP Kit, slides were deparaffinized with xylene and rehydrated in a series of graded ethanols and place in a TBS (pH 7.5) bath for 10 minutes. Next, slides were incubated in a peroxidase quenching solution 3% H_2O_2 in methanol for 10 minutes and treated with a serum blocking solution for 10 minutes. Sections were then incubated with 100 µl of primary antibody for 45 minutes and rinsed with TBS and incubated with 100 µl of biotinylated secondary antibody and rinsed with TBS. Sections were treated with 100 µl of enzyme conjugate and the presence of the enzyme was revealed by incubation of the tissue with the substrate-chromogen solution. Finally, slides were rinsed in dH_2O and mounted with coverslips.

After staining, slides were viewed with differential interference contrast microscopy and photographed. Patterns of immunoreactivity were determined by viewing slides directly and photographs of all sections were then used to support these determinations of distribution and relative staining intensity. Five regions of the tissue were examined: apical tips of the epithelial cells, cytoplasm of the epithelial cells, glands, stroma, and myometrium. Immunocytochemical results were scored for each region in the following manner: 0 = no immunoreactivity, + = low immunoreactivity, ++ = moderate immunoreactivity, and +++ = high immunoreactivity.

Statistics

Immunocytochemical data were analyzed by the nonparametric Kruskal-Wallace test with a significance level set at $p<0.05$.

Results

Validation

Control sections which were incubated without anti-IGF-I/anti-EGF or with non-immune normal rabbit serum exhibited minimal background or no immunostaining. Similar staining distribution and intensity were obtained by both immunocytochemical techniques, thus I will report the results using both techniques, as one data set.

Experimentation

Vitellogenic tube

The luminal epithelium of the tube region (Fig. 3-1), which stains intensely with Alcian blue for glycosaminoglycans (GAGs) contains two types of tall, simple columnar cells: ciliated cells with apical nuclei and non-ciliated microvillous secretory cells with central, or sometimes basal, nuclei (see Palmer and Guillette, 1992). The glands within the tube region are branched acinar. Immunostaining for IGF-I was present in all tissue regions observed: epithelial cells, glands, stroma, and myometrium (Fig. 3-1). Moderate to intense staining was present at the apical tips of the epithelial cells of all stages of vitellogenesis: early, mid, and late vitellogenic animals. Moderate immunostaining throughout the cytoplasm of the epithelial cells was observed in tissue from early vitellogenic animals only. An interesting pattern was noted in the oviductal glands. Moderate immunoreactivity was present in the glands of early-vitellogenic tissue, low immunoreactivity in mid-vitellogenic tissue, and no immunoreactivity in late-vitellogenic tissue.

Low immunoreactivity was observed in the stroma and low to moderate immunoreactivity in the myometrium for all animals.

Epidermal growth factor immunoreactivity was present in the tips of the epithelial cells in early (moderate to high), mid (none to low), and late (none to low) vitellogenic animals. In the cytoplasm of the epithelial cells, moderate immunoreactivity was observed in early vitellogenic tissue only. A staining pattern similar to the one for IGF-I immunoreactivity was observed in the oviductal glands. Moderate immunoreactivity was observed in early vitellogenic tissue, low immunoreactivity in mid vitellogenic tissue, and no immunoreactivity in late vitellogenic tissue. Immunoreactivity was absent or very low in the stroma and low to moderate in all animals observed.

Vitellogenic fiber uterus

The luminal epithelium of the fiber-secreting region of the uterus (Fig. 3-2) consists of simple columnar cells similar to those described for the tube region. However, these cells are lower in height and stain less intensely with Alcian blue. The glands are branched tubular. No immunoreactivity for IGF-I was observed at the apical tips of the epithelial cells in all but three animals. Of those three animals, one exhibited low immunoreactivity, one moderate immunoreactivity, and one high immunoreactivity at the tips of the epithelial cells. Likewise no immunoreactivity was observed throughout the cytoplasm of the epithelial cells in any animals except one early vitellogenic animal which exhibited moderate immunoreactivity. Inconclusive results were obtained for the glands of the fiber uterus. Early and mid vitellogenic animals exhibited a wide range of immunoreactive intensities in the glands (no staining, low, moderate, high), whereas no immunoreactivity was observed in the glands of late vitellogenic animals. Immunostaining results from this region of the oviduct were difficult to

interpret due to high levels of background (nonspecific) staining in the control slides. This high background staining may have resulted from either high levels of endogenous peroxidase activity or high levels of avidin within this oviductal region. In early vitellogenic animals, immunoreactivity was absent in the stroma, whereas low immunoreactivity was observed in this area in mid vitellogenic animals, and moderate immunoreactivity in late vitellogenic animals. In the myometrium, immunoreactivity varied from the absence of staining to high-intensity staining.

Low EGF immunoreactivity was present in the apical tips of the epithelial cells of early and mid vitellogenic animals and absent in late vitellogenic animals. Low immunoreactivity was observed throughout the cytoplasm of the epithelial cells of early vitellogenic tissue only. Low to moderate immunoreactivity was observed in the glands of early vitellogenic tissue, whereas no to very low immunoreactivity was observed in mid and late vitellogenic animals. No to very low immunoreactivity was observed in the stroma of all tissue examined and immunoreactivity in the myometrium was low to moderate.

Vitellogenic calcium uterus

The luminal epithelium of the calcium-secreting region of the uterus (Fig. 3-3) consists of low columnar cells as described for the fiber uterus; however, very few cells stain positively with Alcian blue. The endometrial glands of this posterior uterine region are branched tubular. Insulin-like growth factor-I immunoreactivity was observed in the tips of the epithelial cells in tissue from early (low), mid (low), and late (moderate) vitellogenic animals. Throughout the cytoplasm, immunoreactivity is moderate in early and low in both mid and late vitellogenic animals. In the glands, immunoreactivity is similar in all three groups, low to moderate. In the

stroma, immunoreactivity is low in mid vitellogenic animals and absent in both early and late vitellogenic animals. In the myometrium, immunoreactivity is low or absent in early vitellogenic animals and low to moderate in mid and late vitellogenic animals.

Low EGF immunoreactivity was observed both in the apical tips and throughout the cytoplasm of the epithelial cells of early, mid, and late vitellogenic animals. Low immunoreactivity was observed in the glands of early and late vitellogenic animals, whereas in mid vitellogeinc animals moderate immunoreactivity was observed. No to very low immunoreactivity was observed in the stroma. In the myometrium, no immunoreactivity was observed in tissue from early or mid vitellogenic animals and low to moderate immunoreactivity was observed in mid vitellogenic animals.

Gravid tube

In the tube (Fig. 3-4), IGF-I immunoreactivity was present in the apical tips of the epithelial cells: low immunoreactivity for early and mid gravid animals and high immunoreactivity for late gravid animals. Immunostaining was absent throughout the cytoplasm of the epithelial cells in all tissues examined. Oviductal glands in late gravid animals exhibited moderate to high staining whereas low immunoreactivity was observed in the glands of early and mid gravid animals. No or very low immunoreactivity was observed in the stroma of all tissue examined. In the myometrium, low immunoreactivity was observed in early and mid gravid animals and moderate immunoreactivity was observed in late gravid animals.

Low EGF immunoreactivity was observed in the apical tips of the epithelial cells and no immunoreactivity was observed throughout the

cytoplasm of these cells. Low to moderate immunoreactivity was present in the glands in early gravid animals, but was absent in the glands of both mid and late gravid animals. Epidermal growth factor immunoreactivity was also either absent or very low in the stroma of all tissue examined. Low immunoreactivity was observed in the myometrium of all animals.

Gravid fiber uterus

In the fiber uterus (Fig. 3-5) immunoreactivity was low in the apical tips of epithelial cells in early and mid gravid animals and low to moderate in late gravid animals. No to low immunoreactivity was observed throughout the epithelial cells. Glands exhibited low immunoreactivity in early gravid animals and moderate immunoreactivity in mid gravid animals. In late gravid animals, immunoreactivity in the glands ranged from very low to high with most of the glands exhibiting low to moderate staining. Immunoreactivity in the stroma was low in early and mid gravid animals and moderate in late gravid animals. In the myometrium, immunoreactivity was either absent or very low in all animals.

Epidermal growth factor immunoreactivity was absent or very low in the apical tips of the epithelial cells and absent throughout the cytoplasm of the epithelial cells in the fiber uterus. In the glands, low to moderate staining was observed in early and mid gravid animals. In late gravid animals, immunoreactivity ranged from low to high with most glands exhibiting moderate-intensity staining. Immunostaining in the myometrium was either very low or absent in all animals.

Gravid calcium uterus

In the calcium uterus (Fig. 3-6) IGF-I immunoreactivity was low in early and mid gravid animals and moderate to high in late gravid animals. Immunoreactivity throughout the cytoplasm of the epithelial cells was very

low in early gravid animals, low in mid gravid animals, and moderate in late gravid animals. Immunoreactivity was low to moderate in the glands, and it was absent in the stroma of early and mid gravid animals and low to moderate in late gravid animals. In the myometrium low immunoreactivity was observed in all animals.

Epidermal growth factor immunoreactivity was observe at the apical tips of the epithelial cells in early (low), mid (low to moderate), and late (low to moderate) gravid animals, and immunoreactivity throughout the cytoplasm of the epithelial cells was low in all animals. In the glands, immunoreactivity was low to moderate in all animals, and it was absent or very low in both the stroma and the myometrium.

Post-oviposition tube

Insulin-like growth factor-I immunoreactivity in the apical tips of the epithelial cells of the tube (Fig. 3-7) was low to moderate in early post-oviposition, mid post-oviposition, and late post-oviposition animals, whereas immunoreactivity throughout the cytoplasm of the epithelial cells was absent to low. In early post-oviposition and late post-oviposition animals, immunoreactivity in the glands was low whereas in mid post-oviposition animals it was low to moderate. Low immunoreactivity was observed in the stroma of early post-oviposition and mid post-oviposition animals, and no immunoreactivity was observed in the late post-oviposition animals. In the myometrium, immunoreactivity varied from absent to moderate in all tissues examined.

Moderate EGF immunoreactivity was observed in the apical tips of the epithelial cells in early post-oviposition and mid post-oviposition animals, but it was absent in late post-oviposition animals. Likewise, low to moderate immunoreactivity was present throughout the cytoplasm of the epithelial

cells in early post-oviposition and mid post-oviposition animals but was absent in late post-oviposition animals. Moderate immunoreactivity was present in the glands of early post-oviposition and mid post-oviposition animals, whereas low immunoreactivity was observed in the glands of late post-oviposition animals. Immunoreactivity was absent or very low in the stroma of all animals. In the myometrium, moderate immunoreactivity was present in early post-oviposition, low immunoreactivity in mid post-oviposition, and no immunoreactivity in late post-oviposition animals.

Post-oviposition fiber uterus

In the fiber uterus (Fig. 3-8), low IGF-I immunoreactivity was observed in early post-oviposition and mid post-oviposition animals, and low to moderate immunoreactivity was observed in late post-oviposition animals. Immunoreactivity throughout the cytoplasm of the epithelial cells was absent in early post-oviposition and mid post-oviposition and low to moderate in late post-oviposition animals. In the glands, low to moderate immunoreactivity was observed in all animals. In both the stroma and the myometrium, immunoreactivity was absent or low in all tissue examined.

Moderate EGF immunoreactivity was observed in the apical tips of the epithelial cells in early post-oviposition animals, whereas immunoreactivity in mid post-oviposition and late post-oviposition animals was absent or very low. Low immunoreactivity was present in early post-oviposition animals throughout the cytoplasm of the epithelial cells but absent in both mid post-oviposition and late post-oviposition animals. In the glands, moderate immunoreactivity was observed in early post-oviposition, low immunoreactivity in mid post-oviposition, and low, moderate, and high immunoreactivity was observed in the late post-oviposition animals.

Immunoreactivity was absent in the stroma and low in the myometrium of all animals examined.

Post-oviposition calcium uterus

In the calcium uterus (Fig. 3-9), low to moderate IGF-I immunoreactivity was observed in the apical tips of the epithelial cells in early post-oviposition and mid post-oviposition animals. In late post-oviposition animals, some tips exhibited no immunoreactivity, some moderate immunoreactivity, and some high immunoreactivity. Low immunoreactivity was present throughout the epithelial cells in early post-oviposition and mid post-oviposition, whereas moderate immunoreactivity was observed in late post-oviposition animals. Low immunoreactivity was present in the glands of early post-oviposition animals, whereas moderate immunoreactivity was observed in the glands of mid post-oviposition and late post-oviposition animals. Immunoreactivity was absent or very low in the stroma and low in the myometrium for all animals examined.

Low to moderate EGF immunoreactivity was observed in the apical tips of the epithelial cells in early post-oviposition and mid post-oviposition animals, but absent in late post-oviposition animals. Immunostaining throughout the cytoplasm of the epithelial cells was low to moderate in early post-oviposition and mid post-oviposition, but absent in late post-oviposition animals. In the glands, low to moderate immunoreactivity was present in early post-oviposition and mid post-oviposition animals and either absent or very low in late post-oviposition animals. Epidermal growth factor immunoreactivity was low in the stroma of early post-oviposition and absent in mid post-oviposition and late post-oviposition animals. In the myometrium, moderate immunoreactivity was observed in early post-

oviposition, low immunoreactivity in mid post-oviposition, and no immunoreactivity in late post-oviposition animals.

Nonreproductive tube

In six of the nine nonreproductive animals examined, no IGF-I or EGF immunoreactivity was observed in the tube region (Fig. 3-10). In one animal, low immunoreactivity in the apical tips of the epithelial cells, in the glands, and in the myometrium was observed. In another animal, only IGF-I immunoreactivity was present and it was observed in the apical tips of the epithelial cells and in the glands. In one animal, IGF-I immunoreactivity was high in the apical tips of the epithelial cells, low throughout the cytoplasm of the epithelial cells, moderate in the glands, and low in both the stroma an myometrium.

Nonreproductive fiber uterus

In four of the nine nonreproductive animals studied, no IGF-I or EGF immunoreactivity was observed in the fiber uterus (Fig. 3-11). In two of the nine animals, immunoreactivity was absent except for low-intensity immunostaining for both peptides in some of the oviductal glands. In one animal, low IGF-I immunoreactivity was observed in the apical tips of the epithelial cells and in the glands, and EGF immunoreactivity was present in the apical tips of the epithelial cells and in the myometrium. In another animal, moderate IGF-I immunoreactivity and low EGF immunoreactivity were observed in the glands. In the last animal, for IGF-I, high immunoreactivity was present in the apical tips of the epithelial cells, moderate immunoreactivity throughout the cytoplasm of the epithelial cells, high immunoreactivity in the glands, no immunoreactivity in the stroma and moderate immunoreactivity in the myometrium. For EGF, this animal exhibited high immunoreactivity in the glands only.

Nonreproductive calcium uterus

In seven of the nine nonreproductive animals studied, immunoreactivity was absent for both IGF-I and EGF in the calcium uterus (Fig. 3-12). For one animal, low immunoreactivity for IGF-I was present in the apical tips of the epithelial cells, throughout the cytoplasm of the epithelial cells, and in the glands. Low immunoreactivity for EGF was observed only in the glands in this animal. In another animal, high immunoreactivity for IGF-I was present in the apical tips of the epithelial cells, moderate immunoreactivity throughout the cytoplasm of the epithelial cells, moderate immunoreactivity in the glands, no immunoreactivity in the stroma, and moderate immunoreactivity in the myometrium. For EGF, this same animal exhibited moderate immunoreactivity in the apical tips of the epithelial cells, low immunoreactivity throughout the cytoplasm of the epithelial cells, moderate immunoreactivity in the glands, no immunoreactivity in the stroma, and low immunoreactivity in the myometrium.

Statistics

Statistically significant relationships among gross reproductive stage (nonreproductive, vitellogenic, gravid, and post-oviposition) and immunocytochemical ranking for IGF-I and EGF in the three regions of the oviduct (tube, fiber uterus, and calcium uterus) are presented in Table 3-1. Statistically significant relationships among reproductive stage (nonreproductive, early vitellogenic, mid vitellogenic, late vitellogenic, early gravid, mid gravid, late gravid, early post-oviposition, mid post-oviposition, and late post-oviposition) and immunocytochemical ranking for IGF-I and

EGF in the three regions of the oviduct (tube, fiber uterus, and calcium uterus) are displayed in Table 3-2.

Table 3-1. Statistically significant relationships among gross reproductive stage and immunocytochemical ranking for IGF-I and EGF in the oviduct of the American alligator, *A. mississippiensis*. (V=vitellogenic, G=gravid, PO=post-oviposition, NR=nonreproductive). All comparisons were made but only those found to be significantly different are reported here.

Growth Factor	Oviductal Region	Cellular Region	Tied P Value	Immunocytochemical Ranking
IGF	tube	myometrium	0.0486	V > PO > G > NR
IGF	fiber	stroma	0.0229	G > V > PO > NR
IGF	calcium	gland	0.0483	V > PO > G >NR
EGF	tube	myometrium	0.0336	V > PO > G >NR
EGF	tube	cytoplasm	0.0338	PO > V > G = NR
EGF	tube	gland	0.0390	PO > V > G > NR
EGF	fiber	myometrium	0.0059	V > PO > G >NR

Discussion

Insulin-like growth factor I (IGF-I)-like and EGF-like immunoreactive material are present in the oviduct of the mature, female American alligator, *A. mississippiensis*, throughout the reproductive cycle. Positive immunostaining for these peptide growth factors in the alligator oviduct using polyclonal mammalian antibodies suggests a high degree of homology between the reptilian and mammalian forms of these molecules as well as an important functional role for IGF-I and EGF in reptilian reproduction.

Table 3-2. Statistically significant relationships among reproductive stage and immunocytochemical ranking for IGF-I and EGF in the oviduct of the American alligator, *A. mississippiensis*. (EV=early vitellogenic, MV=mid vitellogenic, LV=late vitellogenic, EG=early gravid, MG=mid gravid, LG=late gravid, EPO=early post-oviposition, MPO=mid post-oviposition, LPO=late post-oviposition, NR=nonreproductive) All comparisons were made but only those found to be significantly different are reported here.

Growth Factor	Oviductal Region	Cellular Region	Tied P Value	Immunocytochemical Ranking
IGF	tube	gland	0.0168	EV > LG = MPO > LPO > MG = EG = MV > NR > EPO > LV
IGF	tube	stroma	0.0456	MV > LG = EPO = MPO = LV > EG > NR > EV = MG = LPO
IGF	calcium	stroma	0.0273	MV > LG > LPO > EG = EPO = MPO = NR = EV = LV = MG
EGF	tube	apical tip	0.0057	EV = MPO > EPO > EG >LG = MV > MG >NR > LV = LPO
EGF	tube	cytoplasm	0.0010	EV > EPO = MPO >EG > NR > MV = LV = MG = LG =LPO
EGF	tube	gland	0.0032	MPO > EV = EPO > EG > MV > MG = LPO > NR > LV = LG
EGF	tube	myometrium	0.0100	EPO > EV > MV = MPO > EG > LG > LV > MG > NR > LPO
EGF	fiber	apical tip	0.0259	EPO > EV = MV > EG > MG = LG = MPO > NR > LV = LPO
EGF	fiber	cytoplasm	0.0010	EV > EPO > NR = MV = LV = EG = MG = LG =MPO = LPO
EGF	calcium	cytoplasm	0.0350	MPO > EPO >MV = LV > EG = LG > MG > EV > NR > LPO
EGF	calcium	gland	0.0370	MV > MPO > LG = EPO > LV = EG = MG > NR > EV > LPO
EGF	calcium	stroma	0.0337	MV = EPO > EG > NR = EV = LV = MG = LG = MPO = LPO
EGF	calcium	myometrium	0.0303	MV = EPO > LG = MPO > EV = EG = MG > NR > LV = LPO

Hormonal regulation of the development of the reptilian oviduct, as in other vertebrates, involves the steroid hormones E_2 and P_4. Estradiol-17β secreted by developing follicles serves two basic functions in reptiles. First, E_2 stimulates the liver to synthesize the yolk precursor protein vitellogenin which is then secreted into the blood, carried to the ovaries, and transformed into yolk (Ho et al., 1982). Second, E_2 stimulates growth and differentiation of the reproductive tract in preparation for oviposition (Mead et al., 1981; Jones and Guillette, 1982; Guillette et al, 1991). Estradiol-17β stimulation of oviductal growth in alligators (Forbes, 1938), iguanid lizards (Callard et al., 1972), and snakes (Mead et al., 1981) indicates that estrogens serve a similar function in all reptiles. The additional observation that E_2 alone is not sufficient to restore the oviducts of ovariectomized garter snakes to their full preovulatory state (Mead et al., 1981) suggests that other hormones are essential for normal oviductal growth and development.

Three types of glands are found in the reproductive tract of alligators. Glands found in the tube region of the oviduct are responsible for the secretion of albumen, whereas glands found in the uterus are responsible for either fiber or calcium secretion. These glands are present in greatly reduced numbers during reproductive quiescence. The numbers of glands increase dramatically as follicles begin to grow, suggesting that recruitment of glands is stimulated by E_2 which is secreted in large quantities by growing follicles. These glands are derived from the luminal epithelium of the oviduct. The tube glands form as the tubal epithelium invaginates to form deep pockets lined by secretory cells. The uterine glands form as the uterine epithelium invaginates, but these glands come to lie deep within the stroma in both the fiber uterus and the calcium uterus.

Several interesting immunoreactive staining patterns were observed during the reproductive cycle. Immunoreactivity for both IGF-I and EGF was absent or extremely low in eight of the nine nonreproductive animals studied. In one animal, however, IGF-I immunoreactivity was very intense in all areas except the stroma. Low to moderate EGF immunoreactivity was present in all areas except the stroma where no immunoreactivity was observed. These results were not associated with elevated plasma E_2 or P_4 levels (Guillette et al., 1994) suggesting the regulation of oviductal IGF-I and EGF synthesis by other hormones such as growth hormone. Interestingly, the oviduct of this animal appeared hypertrophied compared to the oviducts of other nonreproductive animals suggesting that, in fact, oviductal growth is stimulated by these growth factors.

During vitellogenesis, in the tube, IGF-I immunoreactivity in the glands was moderate in early vitellogenic animals, low in mid vitellogenic animals, and absent in late vitellogenic animals. These data suggest that the IGF-I in the glands during early vitellogenesis is secreted from the glands. This IGF-I could be involved in oviductal proliferation and differentiation in preparation for egg laying via an autocrine/paracrine pathway. Moderately-intense IGF-I immunoreactivity was present in the apical tips of the epithelial cells throughout vitellogenesis. This IGF-I which appears to be stored in the tips of the epithelial cells could be an important secretory product which is incorporated into the egg albumen. During gravidity, however, IGF-I immunoreactivity in the apical tips of the epithelial cells and in the glands of the tube increased from low in early gravid animals to moderate in mid gravid animals, to high in late gravid animals. Epidermal growth factor immunoreactivity, on the other hand, decreased in the glands during both vitellogenesis and gravidity.

In the fiber uterus, both IGF-I and EGF immunoreactivity increased in the glands from low intensity during early vitellogenic to moderate intensity during mid vitellogenic to high intensity during late vitellogenic. Similarly, IGF-I immunoreactivity in the stroma increased from low in early vitellogenic to moderate in late vitellogenic. In the calcium uterus, IGF-I immunoreactivity appears to increase in the apical tips of the epithelial cells, cytoplasm of the epithelial cells, glands, and stroma from low intensity in early vitellogenic animals to moderately-high intensity in late vitellogenic animals. The myometrium exhibited low immunoreactivity throughout vitellogenesis. It appears, therefore, that increasing immunoreactivity for IGF-I in the endometrium is associated with rising E_2 levels and that IGF-I immunoreactivity in the myometrium is not related to fluctuating E_2 levels. These data are similar to those reported for mammals. Tang et al. (1994) demonstrated that IGF-I immunoreactivity in the luminal and glandular epithelium of human uterine tissue is cycle dependent with greatest immunoreactivity occuring during the late proliferative and early/mid secretory stages of the menstrual cycle. They also reported that IGF-I immunoreactivity was not cycle dependent in the myometrium. No immunoreactivity pattern was apparent for EGF staining in the calcium uterus.

In early post-oviposition animals, IGF-I immunoreactivity in the tube was low in the apical tips of the epithelial cells, cytoplasm of the epithelial cells, glands, stroma, and myometrium. It remained low in the glands and myometrium throughout the post-oviposition period. In the stroma and the cytoplasm of the epithelial cells, IGF-I immunoreactivity disappeared by late post-oviposition, but in the apical tips of the epithelial cells, immunostaining was slightly more intense during late post-oviposition than in early post-

oviposition and mid post-oviposition. EGF immunoreactivity was moderate in the apical tips of the epithelial cells, cytoplasm of the epithelial cells, glands, and myometrium during early post-oviposition and decreased to low in the glands and disappeared in the apical tips of the epithelial cells, in the cytoplasm of the epithelial cells, and in the myometrium by late post-oviposition. Immunoreactivity was low in the stroma throughout the post-oviposition period.

In the fiber uterus, IGF-I immunoreactivity was absent or low in the apical tips of the epithelial cells, cytoplasm of the epithelial cells, stroma, and myometrium during early post-oviposition and disappeared in all areas except the stroma which exhibited low immunoreactivity during late post-oviposition. In the glands, immunoreactivity was low to moderate during early post-oviposition and increased to moderate by late post-oviposition. EGF immunoreactivity was moderate in the apical tips of the epithelial cells and decreased to low in mid post-oviposition and was absent in late post-oviposition. In the glands, immunoreactivity was moderate during mid post-oviposition and varied during late post-oviposition: some glands stained intensely, some moderately, and in some, immunoreactivity was absent. EGF immunoreactivity was low in the cytoplasm of the epithelial cells and the stroma during early post-oviposition and was absent in mid post-oviposition and late post-oviposition. Immunoreactivity in the myometrium was low throughout the post-oviposition period.

In the calcium uterus, IGF-I immunoreactivity was low in all five areas examined during early post-oviposition. Immunoreactivity in the tips of the epithelial cells, in the cytoplasm of the epithelial cells, and in the glands increased from low-moderate during mid post-oviposition to moderate during late post-oviposition immunoreactivity in the stroma and in the

myometrium remained low throughout the post-oviposition period. EGF immunoreactivity was low in all five areas examined during early post-oviposition and mid post-oviposition and was absent during late post-oviposition.

The various patterns of IGF-I and EGF immunoreactivity in different oviductal regions during the reproductive cycle in the alligator suggest that the hormonal regulation of their synthesis, storage, and secretion is complex. Patterns of immunoreactivity can not be explained based on plasma E_2 and P_4 concentrations alone because intense immunoreactivity is present in tissues exhibiting vastly different circulating levels of both of these hormones. Other hormones such as growth hormone and various other polypeptide growth factors likely play important roles in IGF-I and EGF regulation in the alligator oviduct.

Figure 3-1. Immunocytochemical localization of IGF-I and EGF in the tube of a mid vitellogenic alligator (400X)A: Basic histology: epithelium (e), gland (g), stroma (s), and myometrium (m). B: Experimental tissue incubated with polyclonal anti-human IGF-I rabbit antiserum. C: Experimental tissue incubated with polyclonal anti-mouse EGF rabbit antiserum. D: Control tissue incubated with non-immune normal rabbit serum.

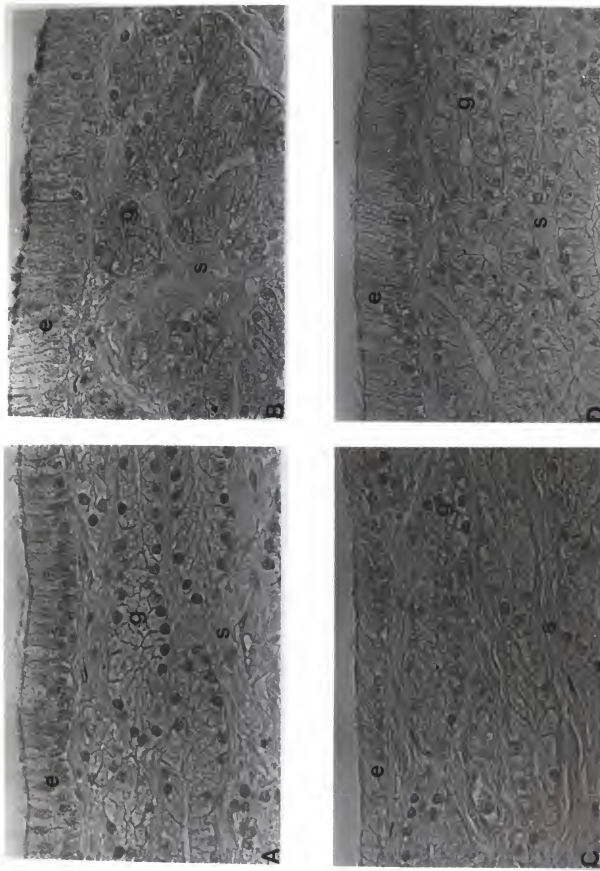
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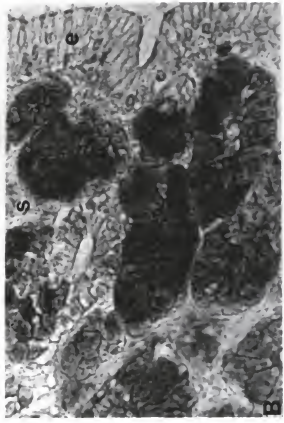
Figure 3-2. Immunocytochemical localization of IGF-I and EGF in the fiber uterus of a mid vitellogenic alligator (400X)A: Basic histology: epithelium (e), gland (g), stroma (s), and myometrium (m). B: Experimental tissue incubated with polyclonal anti-human IGF-I rabbit antiserum. C: Experimental tissue incubated with polyclonal anti-mouse EGF rabbit antiserum. D: Control tissue incubated with non-immune normal rabbit serum.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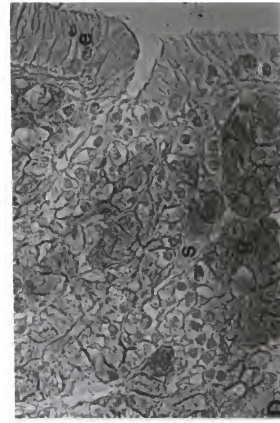
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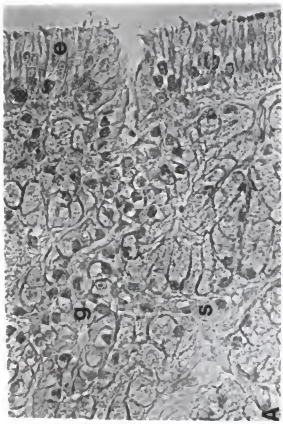
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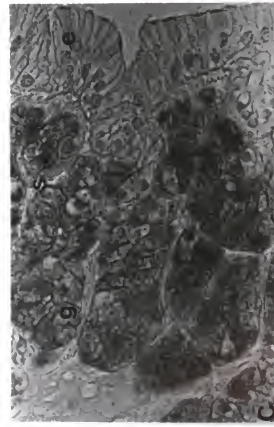
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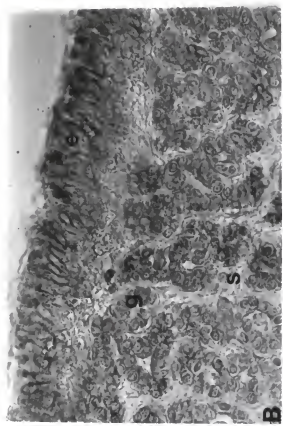
Figure 3-3. Immunocytochemical localization of IGF-I and EGF in the calcium uterus of a mid vitellogenic alligator (400X)A: Basic histology: epithelium (e), gland (g), stroma (s), and myometrium (m). B: Experimental tissue incubated with polyclonal anti-human IGF-I rabbit antiserum. C: Experimental tissue incubated with polyclonal anti-mouse EGF rabbit antiserum. D: Control tissue incubated with non-immune normal rabbit serum.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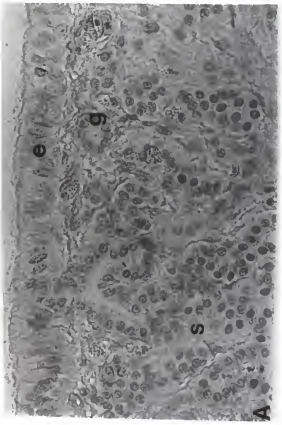
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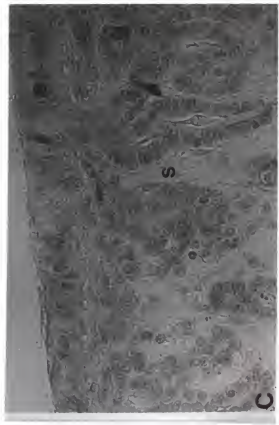
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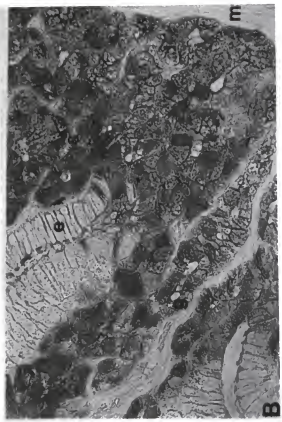
Figure 3-4. Immunocytochemical localization of IGF-I and EGF in the oviductal tube of a late gravid alligator (200X)A: Basic histology: epithelium (e), gland (g), stroma (s), and myometrium (m). B: Experimental tissue incubated with polyclonal anti-human IGF-I rabbit antiserum. C: Experimental tissue incubated with polyclonal anti-mouse EGF rabbit antiserum. D: Control tissue incubated with non-immune normal rabbit serum.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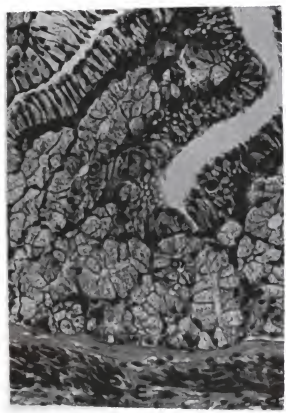

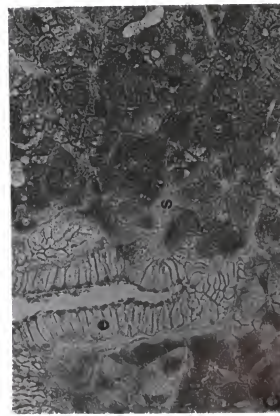
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Figure 3-5. Immunocytochemical localization of IGF-I and EGF in the fiber uterus of a late gravid alligator (400X)A: Basic histology: epithelium (e), gland (g), stroma (s), and myometrium (m). B: Experimental tissue incubated with polyclonal anti-human IGF-I rabbit antiserum. C: Experimental tissue incubated with polyclonal anti-mouse EGF rabbit antiserum. D: Control tissue incubated with non-immune normal rabbit serum.

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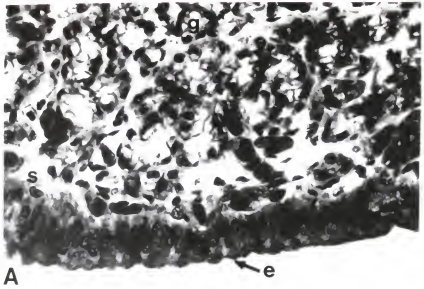
Figure 3-6. Immunocytochemical localization of IGF-I and EGF in the calcium uterus of a late gravid alligator (200X)A: Basic histology: epithelium (e), gland (g), stroma (s), and myometrium (m). B: Experimental tissue incubated with polyclonal anti-human IGF-I rabbit antiserum. C: Experimental tissue incubated with polyclonal anti-mouse EGF rabbit antiserum. D: Control tissue incubated with non-immune normal rabbit serum.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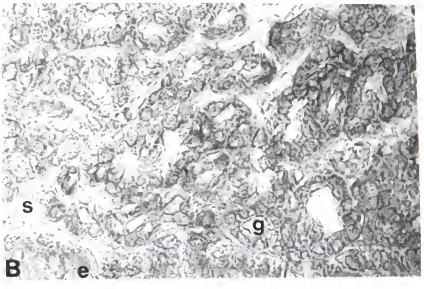
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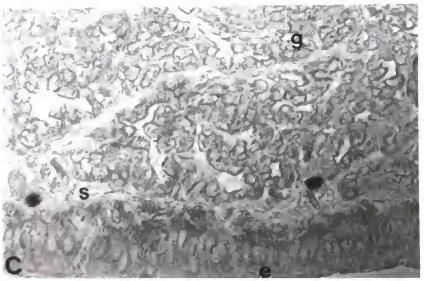
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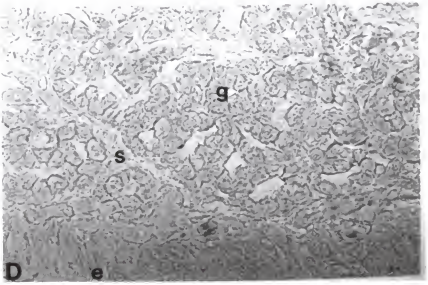
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Figure 3-7. Immunocytochemical localization of IGF-I and EGF in the oviductal tube of a mid post-oviposition alligator (200X)A: Basic histology: epithelium (e), gland (g), stroma (s), and myometrium (m). B: Experimental tissue incubated with polyclonal anti-human IGF-I rabbit antiserum. C: Experimental tissue incubated with polyclonal anti-mouse EGF rabbit antiserum. D: Control tissue incubated with non-immune normal rabbit serum.

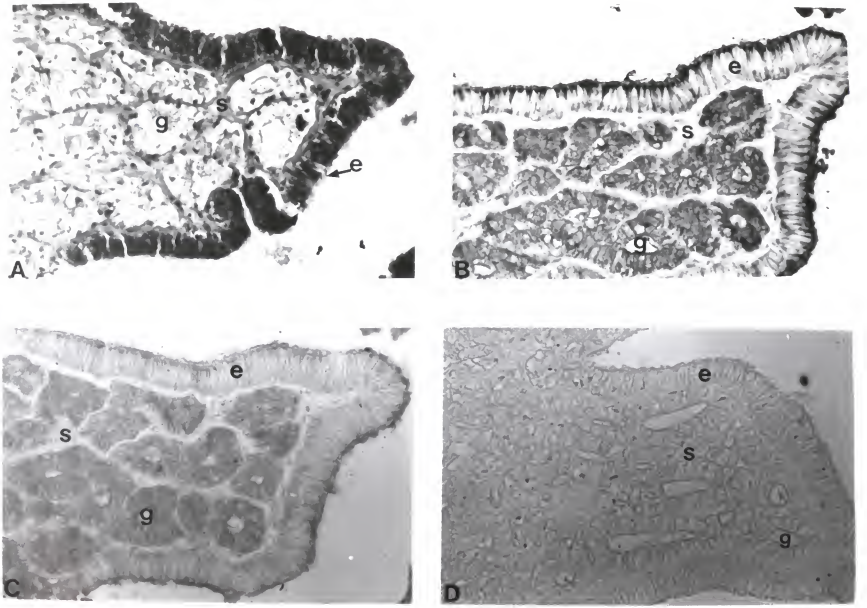
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Figure 3-8. Immunocytochemical localization of IGF-I and EGF in the fiber uterus of a mid post-oviposition alligator (200X)A: Basic histology: epithelium (e), gland (g), stroma (s), and myometrium (m). B: Experimental tissue incubated with polyclonal anti-human IGF-I rabbit antiserum. C: Experimental tissue incubated with polyclonal anti-mouse EGF rabbit antiserum. D: Control tissue incubated with non-immune normal rabbit serum.

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Figure 3-9. Immunocytochemical localization of IGF-I and EGF in the calcium uterus of a mid post-oviposition alligator (200X)A: Basic histology: epithelium (e), gland (g), stroma (s), and myometrium (m). B: Experimental tissue incubated with polyclonal anti-human IGF-I rabbit antiserum. C: Experimental tissue incubated with polyclonal anti-mouse EGF rabbit antiserum. D: Control tissue incubated with non-immune normal rabbit serum.

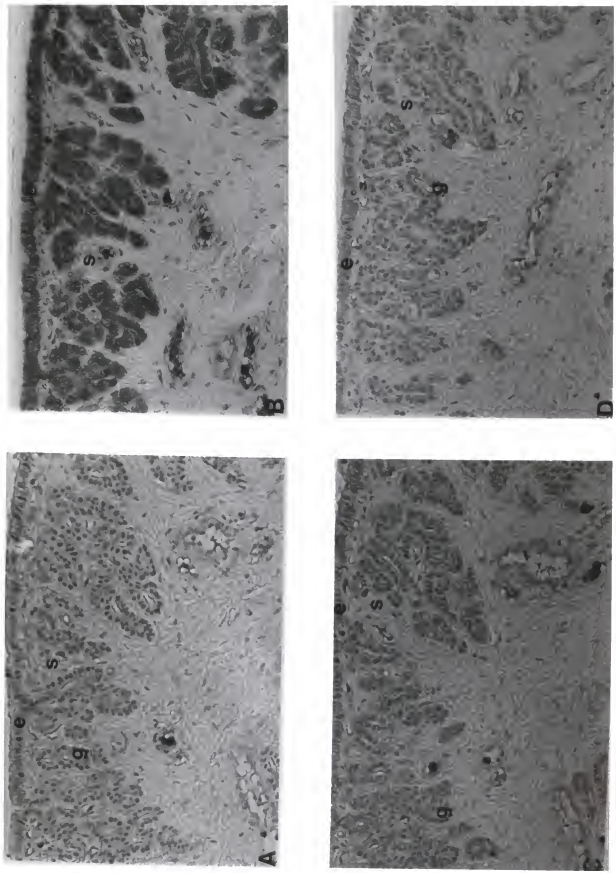
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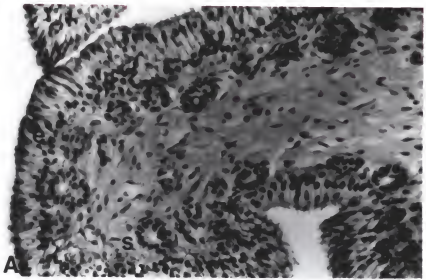
Figure 3-10. Immunocytochemical localization of IGF-I and EGF in the oviductal tube of a nonreproductive alligator (200X)A: Basic histology: epithelium (e), gland (g), stroma (s), and myometrium (m). B: Experimental tissue incubated with polyclonal anti-human IGF-I rabbit antiserum. C: Experimental tissue incubated with polyclonal anti-mouse EGF rabbit antiserum. D: Control tissue incubated with non-immune normal rabbit serum.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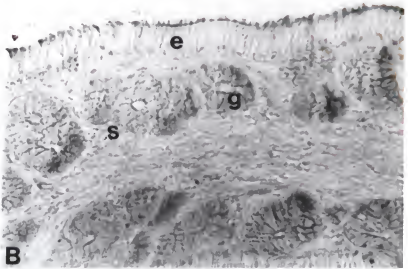
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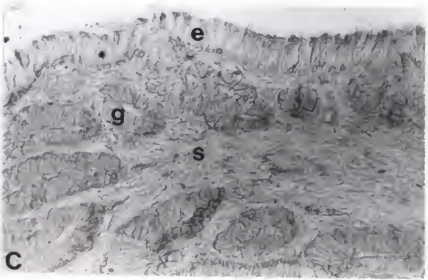
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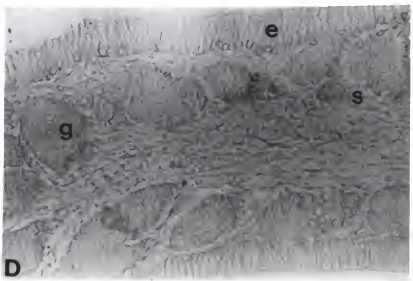
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Figure 3-11. Immunocytochemical localization of IGF-I and EGF in the fiber uterus of a nonreproductive alligator (200X)A: Basic histology: epithelium (e), gland (g), stroma (s), and myometrium (m). B: Experimental tissue incubated with polyclonal anti-human IGF-I rabbit antiserum. C: Experimental tissue incubated with polyclonal anti-mouse EGF rabbit antiserum. D: Control tissue incubated with non-immune normal rabbit serum.

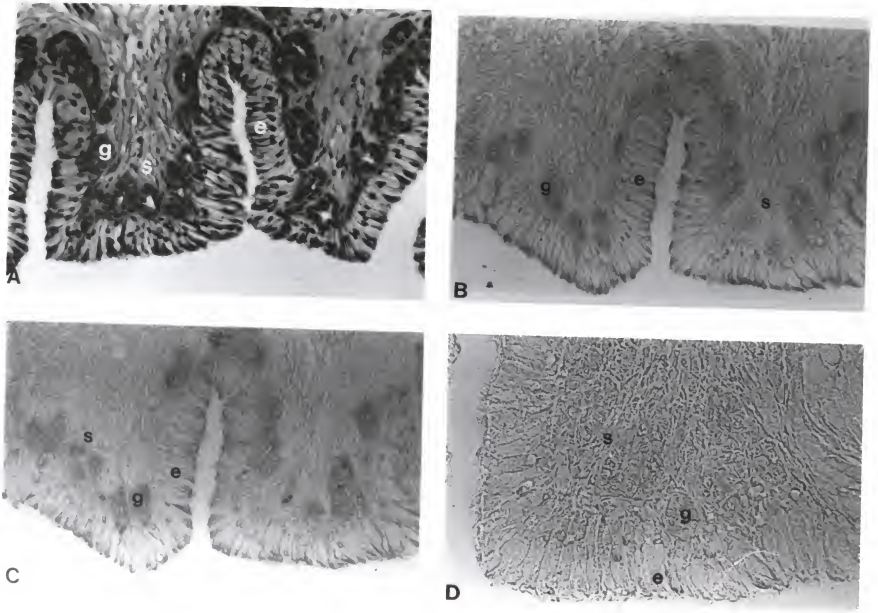
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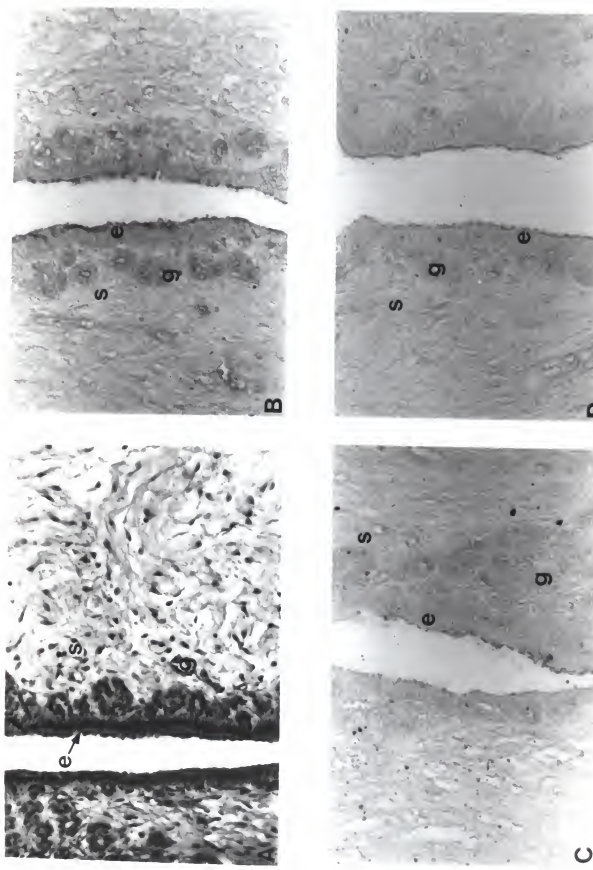
Figure 3-12. Immunocytochemical localization of IGF-I and EGF in the calcium uterus of a nonreproductive alligator (200X)A: Basic histology: epithelium (e), gland (g), stroma (s), and myometrium (m). B: Experimental tissue incubated with polyclonal anti-human IGF-I rabbit antiserum. C: Experimental tissue incubated with polyclonal anti-mouse EGF rabbit antiserum. D: Control tissue incubated with non-immune normal rabbit serum.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CHAPTER 4
PLASMA IGF-I CONCENTRATIONS AND OVIDUCTAL IGF-I MESSENGER RNA IN THE AMERICAN ALLIGATOR *ALLIGATOR MISSISSIPPIENSIS* AND THEIR ASSOCIATION WITH PLASMA ESTRADIOL-17β CONCENTRATIONS

Introduction

The structural complexity of the IGF-I gene provides several sites for potential gene regulation within the biosynthetic pathway resulting in the functional protein product. Insulin-like growth factor I acts through endocrine, autocrine, and paracrine pathways to regulate cellular growth and differentiation. The focus of this study is to begin to examine the interaction of the endocrine and the autocrine/paracrine pathways of IGF-I action during the reproductive cycle of the American alligator, *Alligator mississippiensis*.

In order to begin to elucidate the role that IGF-I plays in reptilian reproduction, it is necessary to study both the endocrine and autocrine/paracrine aspects of this growth factor. This study involved measuring circulating IGF-I concentrations in alligators exhibiting various reproductive states (nonreproductive, vitellogenic, gravid, and post-oviposition) using a specific radioimmunoassay and determining amounts of IGF-I mRNA in the three major functional regions of the oviduct (tube, fiber uterus, and calcium uterus) from nonreproductive, vitellogenic, and gravid animals. I predicted that circulating IGF-I levels would not correlate with circulating E_2 levels since plasma IGF-I originates from the liver under the direct stimulation of GH (Hall and Sara, 1983). In contrast, I hypothesized that

IGF-I mRNA levels in the oviduct should be highest during vitellogenesis when circulating plasma E_2 concentrations are highest since uterine IGF-I is regulated by E_2 in mammals.

Materials and Methods

Specimens

Fifty-seven mature, female alligators (*A. mississippiensis*) were captured from several lakes (Griffin, Orange, Okeechobee) in central Florida (Permit #W88063). Initial blood samples were collected within 15 minutes of capture. Within 24 hours of capture, the animals were anesthetized with 20 mg/kg sodium pentobarbital, and their oviducts were surgically removed. Animals were staged based on gross reproductive state as either non-reproductive (n=15), vitellogenic (n=13), gravid (n=11), or post-oviposition (n=16). Tissues from the three major functional regions of the reproductive tract (tube, fiber-secreting uterus, and calcium-secreting uterus) were dissected from 5 nonreproductive, 7 vitellogenic, and 5 gravid animals and immediately flash frozen in liquid nitrogen and stored at -70^{0}C.

Radioimmunoassay

Sample extraction was based on Daughaday et al. (1980). For each sample, 100 µl of plasma was extracted with 400 µl of acid ethanol (87.5% absolute ethanol, 12.5% 2N hydrochloric acid), vortexed, incubated for 30 minutes at room temperature, and centrifuged at 3,000 g for 10 minutes at 4^{0}C. Validation of the assay was accomplished with both an internal standard curve and a plasma dilution curve (Fig. 4-1). For the six points in the plasma internal standard curve, 100 µl of the supernatant was aliquotted in duplicate

and the samples were spiked with 0, 0.039, 0.156, 0.313, 0.625, 1.25, or 2.5 pg of IGF-I standard. For the plasma dilution curve, 0, 10, 25, 50, and 100 µl of the supernatant were aliquotted in duplicate from extracted plasma pools, and total volumes were brought to 100 µl with acid-ethanol. For the nine points in the standard curve, 0.009, 0.019, 0.039, 0.078, 0.156, 0.312, 0.625, 1.25, or 2.5 ng of IGF-I standard was added to each tube. Internal standards and plasma dilutions were extracted with 400 µl acid-ethanol as previously described.

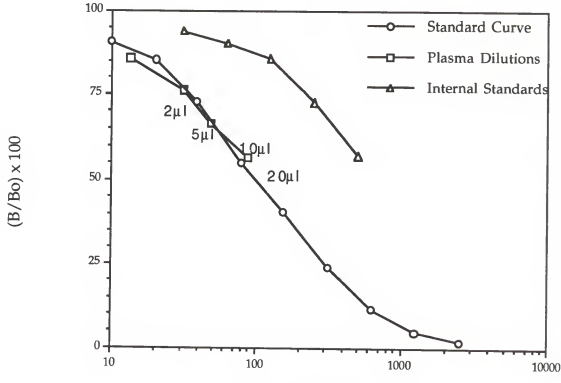


Figure 4-1. Validation for plasma IGF-I radioimmunoassay for the American alligator, *A. mississippiensis*.

RIA buffer (200 mg/L protamine sulfate, 4.14g/L sodium phosphate monobasic, 0.05% Tween 20, 0.02% sodium azide, 3.72g/L EDTA) was added to the standard curve (except the NSB), internal standard curve, plasma dilution, and sample tubes for a total volume of 400µl. RIA buffer (450µl) was added to each of the NSB tubes. IGF-I antibody (50 µl) was added to all tubes

except the NSB tubes, and radiolabeled IGF-I (50 µl) was added to all tubes in addition to two total count tubes. The tubes were vortexed and incubated at 4^0 C for 24 hours. Then, 300 µl of donkey anti-rabbit secondary antibody coated with magnetic separation beads(Amersham International) was added to all tubes except the total count tubes. The tubes were incubated for 10 minutes at room temperature and applied to a magnet for 15 minutes. The supernatant was discarded, the tubes were drained and the pellets were counted on a Beckman 5500B gamma counter. Extraction efficiency was determined by adding approximately 20,000 cpm iodinated IGF-I to 100 µl of pooled plasma and extracting with 400µl acid-ethanol. Following a 30 minute incubation and 10 minute centrifugation, remaining radioactivity was determined. Samples were corrected for an extraction efficiency of 62%.

The animals used in this study were collected as part of an interdisciplinary program examining the reproductive biology of the American alligator in Florida. Consequently, plasma concentrations for estradiol-17β (E_2), and progesterone (P_4) are available for these same animals and will be used with this data set (Guillette et al., 1994).

Dot Blot Analysis

RNA Isolation

RNA was isolated from alligator oviductal tissue using an acid guanidinium thiocyanate-phenol-chloroform extraction procedure (Chomczynski and Sacchi, 1987). One gram of tissue from each oviductal region (tube, fiber uterus, and calcium uterus) of each animal was homogenized in 10 ml chilled denaturing solution (4M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% Sarkosyl, pH 7.0) and 78µl β–mercaptoethanol. Following homogenization, 1 ml 2M sodium acetate (pH

4.0) was added to each sample followed by 10 ml phenol: chloroform: isoamyl alcohol. The samples were mixed by inversion, chilled on ice for 15 minutes and centrifuged at 3000 g at 4^0C for 15 minutes. The top aqueous phase was recovered, 8 ml of isopropanol was added, and samples were incubated overnight at -20^0C. Samples were then centrifuged at 3000 g at 4^0C for 15 minutes, 4 ml denaturing solution and 30µl β–mercaptoethanol was added to the pellets, and the pellets were washed in 4.5 ml isopropanol. Samples were incubated at -20^0C for 4 hours and centrifuged at 3000 g for 15 minutes. The pellets were washed with 10 ml ice-cold 75% ethanol, centrifuged at 3000 g for 15 minutes at 4^0C, and dried in a vacuum dessicator for 15 minutes, resuspended in DEPC-water, and stored at -20^0C.

Loading the Membranes

Serial dilutions of RNA (5, 10, and 20µl) were diluted in 250µl denaturation buffer (50% formamide, 6% formaldehyde, 2% 20mM Tris, pH 7.0), and incubated in a 65^0C water bath for five minutes (Simmen et al., 1990). Pig uterine RNA and yeast RNA were used as positive and negative controls, respectively. 250µl of 20X sodium salt citrate (SSC) was added to each tube which was vortexed and centrifuged briefly for 5-10 seconds. Samples were immobilized on nylon (MSI, magnograph, 0.45 microns) membranes using a microsample filtration unit (Scleicher and Schuell, Keene, NH). The membranes were soaked first in dH_20 and then in 10X SSC prior to loading of the RNA samples. The wells of the filtration unit were prefiltered with 500µl of 10X SSC. The samples were loaded and eluted slowly and the wells were washed with 500µl 20X SSC. The membranes were air dried and baked for 2 hours at 80^0C.

Prehybridization

The membranes were incubated in prehybridization solution (10% 20X SSC, 5% 100X Denhardts, 0.1% NaPPi, 0.1% SDS) and 200µl of 10mg/ml salmon sperm DNA for 2 hours at 40^0C.

Hybridization

The 500 base pair cDNA probe (porcine IGF-I sigf.3, Tavakkol et al., 1988) was labeled using a random priming kit (Stratagene Prime-It II). The cDNA insert was released from a vector (pGEM-4Z), separated on an agarose gel, diluted in sterile dH_20, and heated to 65^0C prior to labeling. The labeled probe was purified through a push column (Stratagene Nuc-trap). One ml TE buffer and 100µl salmon sperm DNA were added to the probe which was denatured by boiling for 10 minutes before being added to the prehybridization solution. The membranes were incubated in the hybridization solution overnight at 40^0C.

Washing

The membranes were washed twice (5 minutes/wash) with 2X SSC (10% 20X SSC, 0.1% SDS, 0.1%NaPPi) and four times (15 minutes/wash) with .4X SSC (0.2% 20X SSC, 0.1% SDS, 0.1% NaPPi).

Statistics

Concentrations of IGF-I were estimated from raw data using the Beckman EIA/RIA program. Resulting RIA data were log transformed to achieve homogeneity of variance and analyzed by one-way ANOVA to determine whether significant variation existed in hormone concentrations among animals of the four reproductive stages. ANOVA was followed by Scheffe's F test with a significance set at $p<0.05$.

Phosphoimaging was used to quantify the dot plot data. Hybridization signals were detected by exposure of the membranes to a phosphoimaging plate for one week. Relative IGF-I mRNA content of the samples was determined using the computer program, Image Quant, and resulting data were analyzed by one-way ANOVA to determine if significant differences existed between relative IGF-I mRNA abundance and reproductive state. ANOVA was followed by Fisher's Protected LSD (least significant difference) test with a significance level at $p<0.05$.

Results

Plasma IGF concentrations were not significantly different among the four gross reproductive states: nonreproductive, vitellogenic, gravid, and post-oviposition ($F = 2.283$; $df = 3$; $p > 0.05$). Interestingly, the nonreproductive animals, which were classified as nonreproductive based on gross morphology, exhibited a tremendous variation in plasma E_2 levels. When these animals were divided into two separate groups, a low E_2 group (<250pg/ml) and a high E_2 group (> 250pg/ml), plasma IGF-I levels were statistically different among groups ($F = 5.645$; $df = 4$; $p < 0.05$) (Fig. 4-2). Two hundred and fifty picograms per milliliter was the lowest level of circulating E_2 in a vitellogenic animal (one with developing ovarian follicles) and, therefore, was used as a measure of reproductive activity to distinguish truely nonreproductive animals from those which had initiated reproductive activity. Insulin-like growth factor-I concentrations in the nonreproductive females exhibiting low E_2 plasma concentrations were significantly higher compared to vitellogenic and post-oviposition animals.

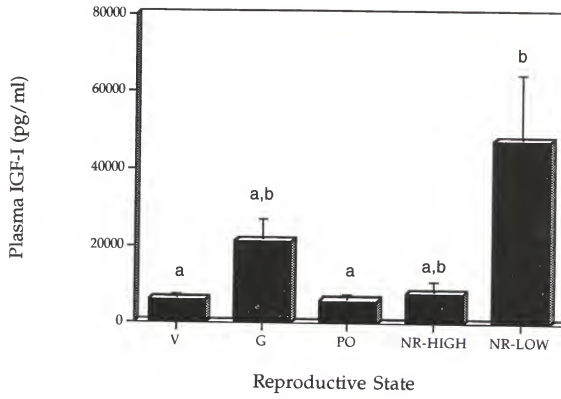


Figure 4-2. Insulin-like growth factor-I concentrations in the plasma of American alligators, *A. mississippiensis*, exhibiting various reproductive stages.

Analysis of the dot blot data revealed a statistical difference in relative abundance of IGF-I mRNA among the three groups (vitellogenic, gravid, and nonreproductive) tested. During the development of the dot blot technique, some oviductal tissue RNA samples were used up; consequently, all of the original samples could not be analyzed. For this reason, all three oviductal regions had to be grouped together for statistical analyses. IGF-I mRNA was detected in all 3 oviductal regions from vitellogenic, gravid, and nonreproductive animals. Tissue from vitellogenic and nonreproductive animals contained significantly less IGF-I mRNA than did tissue from gravid animals (Fig. 4-3).

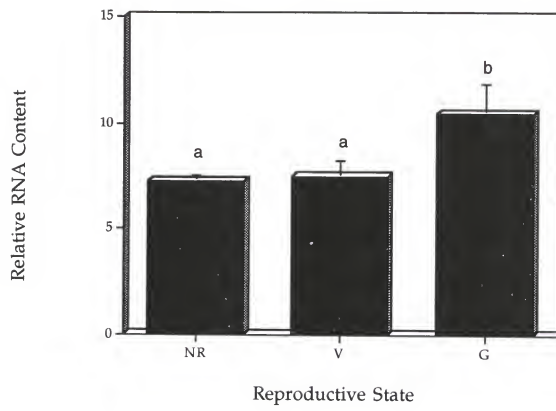


Figure 4-3. Insulin-like growth factor-I mRNA in the oviduct of American alligators, *A. mississippiensis*, exhibiting various reproductive stages.

Discussion

From these data, it is apparent that IGF-I acts via endocrine and autocrine/paracrine pathways in the American alligator, *A. mississippiensis*. Insulin-like growth factor I was detected in the plasma of nonreproductive, vitellogenic, gravid, and post-oviposition animals by a specific radioimmunoassay using a polyclonal rabbit antihuman antibody, and IGF-I mRNA was detected in the tube, fiber uterus, and calcium uterus of the alligator oviduct using a porcine cDNA probe. The detection of IGF-I activity in a reptile using mammalian antibodies and cDNA probes suggests a high degree of homology between reptilian and mammalian IGF-I.

When the nonreproductive animals were divided into two groups based on E_2 levels, significant differences were found in plasma IGF-I concentrations among the five reproductive classifications. Interestingly, concentrations of plasma IGF-I did not correlate with circulating E_2 levels. These data taken together can be used to develop a hypothesis concerning the interaction of the regulation of endocrine versus autocrine/paracrine IGF-I. Nonreproductive animals with high E_2 levels exhibited significantly lower IGF-I levels than did those with low plasma E_2 concentrations Nonreproductive animals that are not preparing for reproduction (those with low E_2) may have increased plasma IGF-I because growth hormone (GH) is stimulating the liver to synthesize and secrete IGF-I. In mice, it has been demonstrated (Murphy and Friesen, 1988) that E_2 inhibits GH stimulation of IGF-I synthesis and release. The same phenomenon could be occurring in alligators. These animals are spending most of their energy on somatic maintenance and growth. Nonreproductive animals, in contrast, which exhibit high circulating E_2 concentrations and low plasma IGF-I concentrations might be preparing for reproduction and, perhaps E_2 inhibits IGF-I synthesis and release from the liver. In these animals, energy must be focused on reproductive activities; consequently, the autocrine/paracrine IGF-I system in the reproductive tract might be of greatest importance at this time.

Insulin-like growth factor mRNA is present in oviductal tissue from all three reproductive stages examined (vitellogenic, gravid, and nonreproductive). Surprisingly, IGF-I mRNA is most abundant in gravid animals when E_2 concentrations are low and P_4 concentrations are elevated. In mammals, high levels of IGF-I mRNA are associated with elevated E_2 levels. For example, in pigs, the highest concentrations of endometrial IGF-I mRNA occur on days 11-12 during early pregnancy when pig blastocysts

initially produce maximal amounts of estrogen (Letcher et al., 1989). Murphy and Friesen (1988) demonstrated that E_2 is a potent stimulator of IGF-I gene expression in rat uteri. Simmen et al. (1990) reported a stimulatory effect of serum P_4 on endometrial IGF-I expression and secretion in the porcine uterus. They also noted the absence of synergism between E_2 and P_4 suggesting that the two steroids may be acting through different mechanisms to regulate the IGF-I gene. The results of this study suggest that P_4 plays a more significant role in the regulation of IGF-I gene regulation than E_2 in reptiles. There are several possible explanations for this apparent difference in IGF-I mRNA regulation between mammals and reptiles.

First, the analysis of alligator IGF-I mRNA was carried out using a heterologous mammalian cDNA probe. At this time, a homologous probe constructed from an alligator cDNA library is not available. Immunocytochemical data (Chapter 2 and 3) suggest a high degree of homology between reptilian IGF-I and mammalian IGF-I at the protein level. Likewise data from this study suggest a high degree of homology between alligator IGF-I mRNA transcripts and their mammalian counterparts. Until the IGF-I gene sequence is determined in the alligator, however, and an appropriate homologous probe can be constructed, results must be interpreted very carefully. As previously described (Chapter 1), using non-homologous mammalian antibodies and probes to localize reptilian IGF-I requires that results be reported in terms of IGF-I-like material since it is not known whether or not the reptilian IGF-I that is being measured is actually IGF-I, IGF-II, or a variant form of these growth factors.

Another possible explanation for the relatively high abundance of mRNA in the gravid alligator oviduct involves the secretion of IGF-I into albumen. Immunocytochemical data show that IGF-I is found in the tips of

the epithelial cells and in the endometrial glands of both vitellogenic and gravid alligator oviducts (Chapter 3). Additionally, IGF-I is present in the albumen of alligator eggs throughout embryonic development (Chapter 6). These data suggest that IGF-I originating from the oviductal glands and epithelial cells is secreted into the albumen. Consequently, an increase in IGF-I mRNA transcripts would be expected during both vitellogenesis and gravidity. It is also plausible that the primary function of IGF-I in the vitellogenic oviduct is mediating estrogen-induced cellular proliferation and differentiation, whereas its presence in the gravid oviduct is a result of its role as a secretory product. This exocrine role for IGF-I represents a novel mechanism for IGF-I action. Endocrine and paracrine/autocrine pathways have been described for IGF-I, and these data suggest an exocrine pathway for this growth factor as well.

CHAPTER 5
GROWTH FACTOR MEDIATION OF ESTRADIOL-17β-INDUCED OVIDUCTAL PROLIFERATION AND DIFFERENTIATION IN THE MEDITERRANEAN GECKO, *HEMIDACTYLUS TURCICUS*

Introduction

Estrogens are essential for normal growth and differentiation of the vertebrate oviduct. However, the observation that estradiol-17β (E_2) initiates little or no response in isolated mammalian uterine cells (Cooke et al., 1986; Cunha and Young, 1992) has led to the hypothesis that paracrine growth factors mediate estrogen-induced proliferation and differentiation of uterine cells. The demonstration that stromal tissue is necessary for estrogen responsiveness in uterine epithelia supports this hypothesis (Cooke et al., 1986; Cunha and Young, 1992). A number of polypeptide growth factors have been implicated as possible paracrine mediators of estrogen-induced oviductal growth including platelet-derived growth factor (Ronnstrand, et al. 1987), transforming growth factor-α, insulin-like growth factor I (Murphy et al., 1988), and epidermal growth factor (Murphy et al., 1989). Much of the *in vivo* mammalian research on growth factor mediation of uterine proliferation and differentiation has focused on EGF. It has been demonstrated that EGF can mediate much of the estrogen-induced uterine growth in ovariectomized mice (Nelson et al., 1990). These data support the hypothesis that rather than acting directly, E_2 exerts its effects via intermediate growth factors, specifically IGF-I and EGF. Administration of E_2 increases uterine IGF-I mRNA in the ovariectomized rat, while decreasing hepatic IGF-I mRNA levels. IGF-I also

potentiates the effects of E_2 on DNA synthesis in the rat uterus in organ culture (Murphy and Ghahary, 1990).

The purpose of this study was to examine the role of polypeptide growth factors in the proliferation and differentiation of the reptilian oviduct. Specifically, this experiment was designed to test the hypothesis that IGF-I and EGF mediate the effects of E_2 in the oviduct of the Mediterranean gecko, *Hemidactylus tursicus*. Adult, female geckos were ovariectomized and implanted with either control, E_2, IGF-I, or EGF pellets in order to test this hypothesis.

Materials and Methods

Specimens

Twenty-three adult female Mediterranean geckos, *H. turcicus*, were captured in Alachua County, Florida during the months of August-October 1992 and July-August 1993. Animals were kept in 78 liter aquaria at 26^{0}C with a 12L:12D photoperiod. Animals were provided with crickets and water *ad libitum*. Prior to ovariectomy, animals were weighed and their snout-vent length (SVL) recorded. Animals were anaesthetized using isoflurane gas (AErrane®, Anaquest, Madison , WI). A small incision was made just to the right of the midline, and the ovaries were removed using an electrocautery unit. The incision was closed using 6-0 silk sutures with a 12 mm needle (American Cyanamid Co., Danbury, CT) and Nexaband® tissue glue (Tri-Point Medical L.D., Raleigh NC). The surgeries were performed with the assistance of Denise Gross, DVM.

Experimental Design

Ovariectomized animals were divided into 4 treatment groups: control (N=6), E_2 (N=6), IGF-I (N=7), and EGF (N=4). Pellets were obtained from Innovative Research of America (IGF-I - 2.5µg/pellet, 14-day release; EGF - 2.5µg/pellet, 14-day release; E_2 - 0.25mg/pellet, 21-day release). After a minimum of 4 weeks, pellets were implanted by making a small incision through the skin and muscle wall left of the ventral midline and using forceps to place the pellet into the body cavity. As with the ovariectomies, silk sutures and tissue glue were used to close the incision. Following a 12-14 day treatment period, the animals were weighed and their SVL recorded. Animals were then euthanized. Blood was collected for radioimmunoassays, and the oviducts and liver were weighed and fixed in Bouin's fixative for 24 hours. Following fixation, tissues were washed in 75% alcohol, dehydrated in a series of graded alcohols, infiltrated and embedded in paraffin, sectioned at 8µm on a rotary microtome, and gelatin-mounted onto slides. Slides were stained with a modified Shorr's staining technique using hematoxylin (nuclear stain), eosin (counterstain), Alcian blue (for glycosaminoglycans), Biebrich scarlett (for blood vessels), fast green (for connective tissue), and orange G (for proteinaceous secretory material).

Morphometrics

Myometrial thickness, epithelial cell height, endometrial thickness, and gland diameter were measured in the uterus. A total of 20 measurements were taken from a single slide using an ocular micrometer for

each parameter for both oviducts of each animal and an average measurement was recorded for each parameter.

Radioimmunoassays

IGF-I and E_2 levels for plasma samples were determined using radioimmunoassays validated for *H. turcicus* plasma. For each animal, both IGF-I and E_2 concentrations were measured in a single plasma sample.

Estradiol-17β

Forty microliter samples were double extracted with 4 ml diethyl ether, vortexed, and placed in a dry-ice-methanol bath. The aqueous phase containing proteins (including IGF-I) froze quickly, and was saved for the IGF-I RIA. The supernatant containing dissolved steroids was poured off into a test tube and air-dried. Extraction efficiency was accomplished by adding 100 µl E_2 radiolabel to a 40 µl plasma pool sample, extracting the sample, removing the supernatnant, air drying the supernatant, adding 4 ml scintillation fluid, and counting the radioactivity. Each sample was analyzed in duplicate and corrected for the extraction efficiency of 70%. Air-dried samples were reconstituted in 500 µl 0.05M PBS (pH 7.5) and divided into two test tubes each containing 250 µl of sample. Standard tubes with 50 µl of a known concentration of radioinert E_2 (12.5, 25, 50, 100, 200, 400, 800, and 1600 pg) were prepared and 250 µl of PBS was added to each tube. An additional 50 µl of PBS was added to each sample tube (for a final volume of 300 µl). After vortexing 100 µl of E_2 antibody which was supplied courtesy of R.I. Butcher, West Virginia University and characterized by T. S. Gross (Guillette et al., 1994) and 100 µl of label (2,4,6,7,16,17-^{3}H estradiol, ~140-170 Ci/mmol) were added to each tube, and tubes were incubated overnight at 4^0C. Two-hundred and fifty microliters of dextran coated charcoal was added to each tube and the

tubes were vortexed and centrifuged for 10 minutes at 3,000 g. The supernatant (500 µl) was removed and added to 5 ml scintillation fluid and counted.

Validation of the assay was accomplished with an internal standard curve (Fig. 5-1). Known amounts (100, 200, 400, 800, 1600 pg) of E_2 were added to 40 µl pooled plasma and measured [Y = -21.32 + .093139X; Y = amount of E_2 measured (pg); X = amount of E_2 added (pg); R^2 = 0.9888]. The minimum detectable dose which was distinguishable from zero was 12.5 pg/ml. Intrassay coefficient of variation was 5.1%.

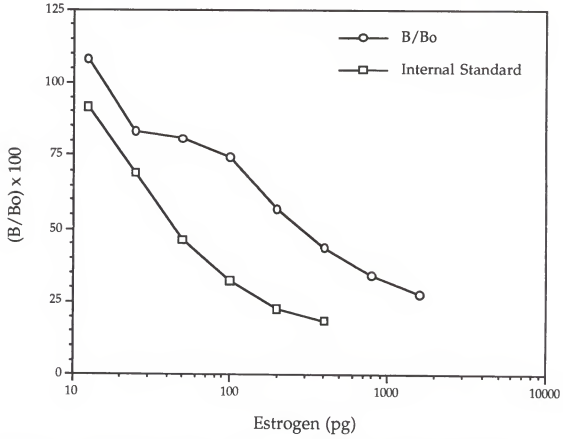


Figure 5-1. Validation for plasma estradiol-17β radioimmunoassy for the Mediterranean gecko, *H. turcicus*.

IGF-I

Human recombinant insulin-like growth factor was used as both a standard and a radioactive tracer (3-[^{125}]iodotyrosyl, Amersham). Rabbit antihuman IGF-I antiserum (lot UBK487, provided by Drs. J.J. Van Wyk and L.L. Underwood through the National Hormone and Pituitary Program was used at a dilution of 1:10,000. Bound-free separation was achieved using a secondary antibody reaction (Amerlex-M, Amersham) and magnetic separation. Validation of the assay was accomplished with an internal standard curve (Fig. 5-2). The aqueous phases remaining from the double ether extractions of the plasma samples were extracted with 160 µl of acid ethanol (87.5% absolute ethanol, 12.5% 2N hydrochloric acid), vortexed, incubated for 30 minutes at room temperature, and centrifuged at 3,000 g for 10 minutes at 4^{0}C (Daughaday, 1980). For the five points in the internal standard curve, 35 µl of the supernatant was aliquotted in duplicate and the samples were spiked with 0.156, 0.312, 0.625, 1.25, and 2.5 ng of IGF-I standard [Y=0.0042991 + 0.82954X; Y = amount of IGF-I measured (pg); X = amount of IGF-I added (pg); R^2=0.9895]. For the nine points in the standard curve, 0.009, 0.019, 0.039, 0.078, 0.156, 0.312, 0.625, 1.25, or 2.5 ng of IGF-I standard (100 µl) was added to each tube. For the plasma samples from the experimental animals, the aqueous phases remaining after the double ether extraction were extracted with 160 µl of acid ethanol, and 35 µl of supernatant was pulled off in duplicate for the assay. RIA buffer was added to the standard curve, except the NSB,, internal standard curve, plasma dilution, and sample tubes for a total volume of 400 µl. Four hundred and fifty microliters of RIA buffer was added to each of the NSB tubes. IGF-I antibody (50 µl) was added to all tubes except the NSB tubes, and labeled IGF-I (50 µl; ~2,000 Ci/mmol) was added to

all tubes in addition to 2 total count tubes. The tubes were vortexed and incubated at 4°C for 24 hours. Then, 300µl of donkey anti-rabbit secondary antibody which is associated with metallic spheres was added to all tubes except the total count tubes. The tubes were incubated for 10 minutes at room temperature and applied to a magnet for 15 minutes. The supernatant was poured off, and the pellet was counted on a Beckman 5500B gamma counter. Extraction efficiency was accomplished by adding 50 µl radiolabeled IGF-I to a 40 µl plasma pool sample, extracting as desribed above, and counting radioactivity. Final IGF-I concentrations were corrected for an extraction efficiency of 63%.

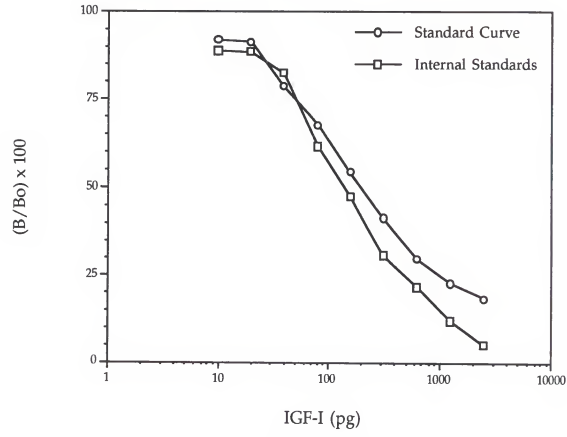


Figure 5-2. Validation for plasma insulin-like growth factor-I radioimmunoassy for the Mediterranean gecko, *H. turcicus*.

Statistical Analyses

Oviductal morphometrical data were analyzed by one-way ANOVA to determine whether significant variation in the four oviductal parameters (epithelial cell height, endometrial thickness, gland diameter, and myometrial thickness) existed among the four experimental treatment groups (control, E_2, IGF-I, and EGF). Log transformation of data was performed in order to achieve homogeneity of variance. ANOVA was followed by Fisher's Protected LSD (least-significant difference) test with a significance level set at $p<0.05$.

Concentrations of E_2 and IGF-I were estimated from raw data using the Beckman EIA/RIA program. Resulting RIA data were analyzed by one-way ANOVA to determine whether significant variation existed in hormone concentrations among the four experimental treatment groups. ANOVA was followed by Fisher's Protected LSD (least-significant difference) test with a significance level set at $p<0.05$.

Results

Estradiol-17β concentrations were undetectable in all experimental animals except those treated with E_2. IGF-I levels were undetectable in all animals. The general morphological characteristics of representative oviducts from each of the four treatment groups are shown (Fig. 5-3). In the control oviducts, epithelial cells are cuboidal and are of two types: non-ciliated secretory and ciliated. Little or no secretory material is present in the lumen. Well-defined capillary beds are located directly beneath the epithelium. A small number of glands are present in the endometrium, but

they appear to contain no secretory material and they have large lumens (Fig. 5-5). The nucleus fills a large portion of the glandular cell as there is very little cytoplasm present. Loose connective tissue is abundant in the endometrium. The myometrium consists of an inner circular layer and an outer longitudinal layer and is surrounded by a poorly defined serosa.

In E_2-treated oviducts, the uterine epithelium consists of non-ciliated secretory and ciliated columnar cells containing central or basal nuclei which stain intensely with Alcian blue, thus identifying an abundance of glycosaminoglycans. Well-developed capillary beds are located directly beneath the epithelium. Large glands fill the endometrium with very little loose connective tissue present. The glands are composed of cells filled with secretory material and possessing basal nuclei. Secretory material is abundant in the oviductal lumen. A well-developed myometrium with an inner circular and outer longitudinal layer is present. Well-developed capillary beds are located between the endometrium and the myometrium, and capillaries are found along the outer layer of the serosa.

In the oviducts obtained from IGF-I-treated animals, the uterine epithelium consists of columnar epithelial cells which stain intensely with Alcian blue indicating an abundance of glycosaminoglycans. Non-ciliated secretory and ciliated cells comprise the epithelium as well as a third distinctive cell type, mucous-secreting goblet cells. The lumen contains an abundance of secretory material. Well-developed capillary beds are located underneath the epithelium. Large glands fill most of the endometrium. The cells of these glands contain an abundance of cytoplasm but do not appear to have a large amount of secretory material. A small, central lumen is present in the center of some glands. The myometrium is well-developed with an

inner circular and outer longitudinal layer. The serosa is also well-developed.

Animals treated with EGF have oviducts in which the epithelium is composed of non-ciliated secretory and ciliated cells. Some secretory material is present in the oviductal lumen, but it is considerably less abundant than in the oviducts obtained from E_2 or IGF-I treated females. Well-defined capillary beds are present directly beneath the epithelium. The myometrium is filled with round glands composed of cells with basal nuclei and a large amount of cytoplasm. Some of the glands have a small central lumen. Capillaries are abundant between the endometrium and myometrium. A well-developed myometrium consisting of an inner circular and outer longitudinal layer is present in addition to a well-defined serosal layer.

Morphometric data are summarized in Fig. 5-5. The heights of the epithelial cells in the animals treated with control pellets were significantly lower than those treated with IGF-I, EGF, or E_2. Additionally, the epithelial cells of IGF-I and EGF-treated oviducts were significantly lower (25%) than those of E_2-treated oviducts, but there was no statistical difference in epithelial cell height between IGF-I and EGF-treated oviducts. Endometrial thickness and gland diameter were significantly lower in control versus E_2, IGF-I, and EGF-treated animals. Furthermore, these two measures were significantly lower in IGF-I-treated and EGF-treated oviducts as compared to E_2-treated oviducts; additionally, IGF-I (for endometrial thickness, 71%, and for gland diameter, 83% of the E_2 response) and EGF-treated (for endometrial thickness, 50%, and for gland diameter, 65% of the E_2 response) oviducts were significantly different from one another with respect to these measurements. No statistical differences were observed in myometrial thickness between treatment groups.

Figure 5-3. Basic histology of the oviduct of the Mediterranean gecko, *H. turcicus* (200X) following treatment with **A.** E_2 **B.** IGF-I **C.** control or **D.** EGF pellets: epithelium (e), gland (g), myometrium (m).

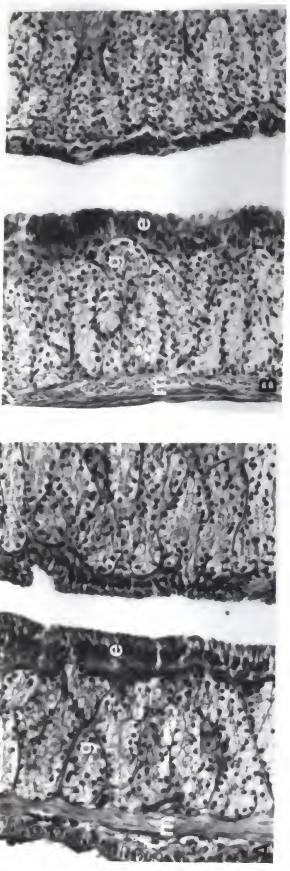
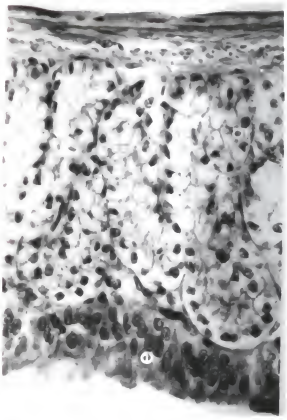
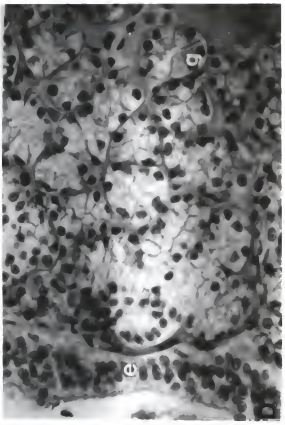
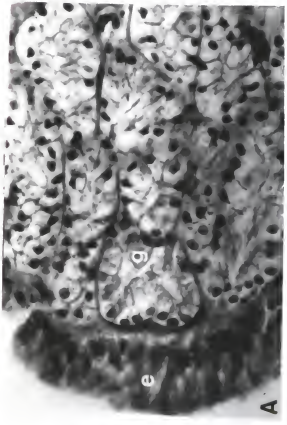

E
B
E
B
E
E
B
E

Figure 5-4. Endometrial glands in the oviduct of the Mediterranean gecko, *H. turcicus* (400X) following treatment with **A.** E_2 **B.** IGF-I **C.** control or **D.** EGF pellets: epithelium (e), gland (g).


e


b
e


b
e
A

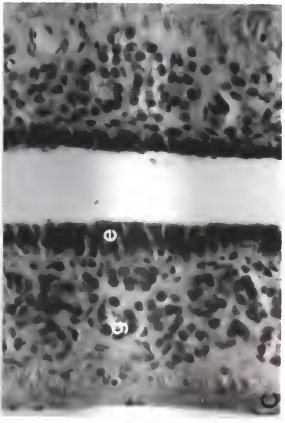
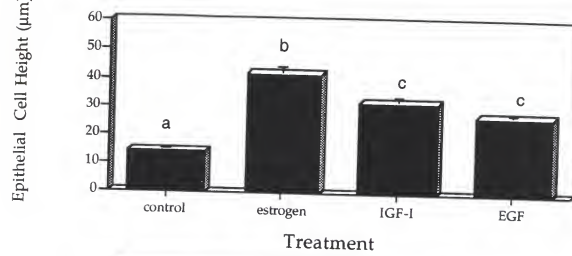
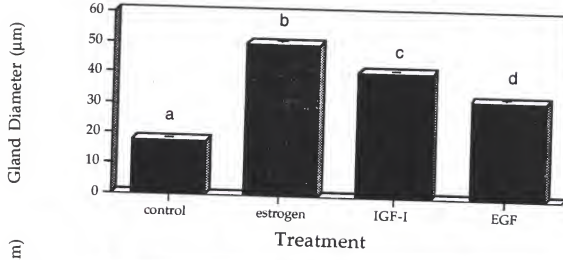
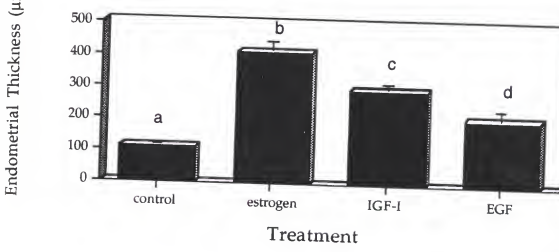

e
b
C

Figure 5-5. Morphometric results of treatment of ovariectomized Mediterranean geckos, *H. turcicus*, with control, E_2, IGF-I, or EGF pellets. epithelial cell height (top graph), gland diameter (middle graph) and endometrial thickness (lower graph).


Epithelial Cell Height (µm)
60
50
40
30
20
10
0
a
b
c
c
control
estrogen
IGF-I
EGF
Treatment


Gland Diameter (µm)
60
50
40
30
20
10
0
a
b
c
d
control
estrogen
IGF-I
EGF
Treatment


Endometrial Thickness (µm)
500
400
300
200
100
0
a
b
c
d
control
estrogen
IGF-I
EGF
Treatment

Discussion

The polypeptide growth factors, IGF-I and EGF, appear to mediate E_2-induced changes in the oviduct of the Mediterranean gecko, *H. turcicus*. The dependence of seasonal oviductal growth and development on ovarian steroids has long been established in reptiles (Noble and Greenberg, 1941; Licht and Hartree, 1971). The actual mechanism by which steroid hormones act on target tissues and elicit biological responses is not clearly understood. Estrogen-induced uterine proliferation in mammals appears to involve a carefully orchestrated activation of genes which are transcribed into biologically active molecules including trans-acting factors and growth factors and their receptors. Both IGF-I and EGF have been associated with stimulation of uterine growth in mammals (Murphy et al., 1987; DiAugustine et al., 1988). The results of this study support the hypothesis that polypeptide growth factors (IGF-I and EGF) act as local mediators of estrogen action in the reptilian oviduct. This study is the first to demonstrate a functional role for these growth factors in the reproductive tract of a reptile, and these data provide strong support for an evolutionary role for IGF-I and EGF in vertebrate oviduct function. While much evidence has accumulated concerning the presence of growth factors such as IGF-I and EGF in non-mammalian vertebrates, little is known of their function.

A number of growth factors and their receptors have been reported in mammalian uterine tissue. The specific functions of these growth factors are largely unknown although many have been suggested as local mediators of E_2 action. McLachlan et al. (1990) proposed a set of four criteria which need to be met in order to establish a biological function for a growth factor. First, the

endogenous ligand should be present in the appropriate site. Both IGF-I and EGF have been identified in the vertebrate reproductive tract. Second, a high affinity receptor for the ligand should be localized to the appropriate tissue. IGF-I and EGF receptors have been identified in the uterus of many mammals (Tang et al., 1994). Third, administration of exogenous growth factor should produce the appropriate physiological response. Nelson et al. (1990) clearly demonstrated the ability of EGF to mediate estrogen-induce oviductal growth in ovariectomized mice. Finally, neutralization of the ligand *in situ* should cause reversal of the expected physiological response. Nelson et al. (1990) reported that an antibody specific for EGF inhibited estrogen-induced uterine and vaginal growth in mice. Data from various laboratories, therefore, strongly indicate important roles for IGF-I and EGF in mammalian oviductal proliferation and differentiation.

The regulatory role of E_2 on IGF-I and EGF expression in reproductive tissues has been documented in several mammalian species. The endocrine control of IGF-I transcription has been examined by ovariectomy and steroid hormone replacement in rats (Croze et al., 1990; Murphy and Ghahary, 1990), mice (Kapur et al., 1992), and pigs (Simmen et al., 1990). Estradiol treatment increased uterine IGF-I transcripts in all species. Stevenson et al. (1994) reported a high correlation between IGF-I mRNA transcription with concentrations of estradiol receptors in both the endometrium and myometrium in sheep, suggesting that in this species, too, IGF-I is directly under the influence of estrogen and, consequently, could act as a mediator of estrogen action. They also reported the presence of high concentrations of IGF-I receptor mRNA in the epithelial cells of the glands and luminal epithelium with lower levels in the stroma and myometrium suggesting a

paracrine role for uterine IGF-I and an interaction between stromal and epithelial cell types in the endometrium.

Stiles et al. (1979) proposed that the IGFs act as progression factors, whereas other growth factors including EGF and platelet-derived growth factor (PDGF) serve as competence factors which initiate the cell cycle and prime the cells to respond to progression factors. The generalized cell cycle consists of four stages: G_1 (gap 1), S (DNA synthesis), G_2 (gap 2), and M (mitosis or meiosis). During the G_1 stage, cells grow, carry out normal metabolism, and duplicate organelles. During the S stage, DNA is replicated, and in the G_2 stage, the cell continues to grow and to manufacture structures such as spindle fibers and centrioles in preparation for cellular division which occurs during the M stage. Cells not actively involved in cellular division remain in a quiescent stage known as G_0. The transition of a cell from the G_0 to the G_1 stage is a major regulatory site for the action of growth factors. *In vitro* studies with mouse fibroblasts have shown that basic fibroblast growth factor (FGFb) and PDGF acted as competence factors enabling cells to pass from G_0 to G_1 but have no further role at later points in the cell cycle. EGF, in contrast, was important during the first part of G_1, and either IGF-I, IGF-II, or insulin was necessary for the final part of G_1 and for entry into the S phase.

The data from this study suggest that growth factors interact to elicit specific cellular responses. Whereas, IGF-I and EGF mediate the actions of E_2, it is clear that these two growth factors alone are not capable of eliciting a complete estrogen response in the gecko oviduct. An interesting model can be developed based on these data. Epidermal growth factor treatment resulted in significantly lower average measurements for both endometrial thickness and gland diameter compared to IGF-I treatment suggesting that IGF-I and

EGF regulate cellular growth and differentiation differently. According to this proposed model, EGF serves as a competence factor and activates the cell cycle by allowing the cell to pass from the G_0 to the G_1 stage or serves as a progression factor during the earliest stages of the G_1 stage. Insulin-like growth factor I acts as a progression factor which stimulate the cell to move through the G_1 stage and on to the S stage. Endometrial thickness and gland diameter measurements were significantly lower in EGF treated oviducts as compare to IGF-I treated oviducts. Estradiol-17β induces synthesis of both IGF-I and EGF which act together to stimulate cell division. When only one of these growth factors is not present then a full response is not possible. When only EGF is present, many of the cells are able to begin the cell cycle, but the absence of progression factors prevents full stimulation of cellular growth which results in overall cellular hypertrophy and hyperplasia. Because IGF-I is involved in regulating a later stage of the cell cycle, IGF-I treatment is able to stimulate a greater cellular response in the oviductal tissue. It is also possible that IGF-I actually up-regulates EGF synthesis. This study clearly demonstrates that IGF-I and EGF are capable of mediating estrogen-induced oviductal proliferation and differentiation in the gecko oviduct. These data also suggest that other hormones or growth factors are involved in this process since administration of either IGF-I or EGF did not elicit a full estrogenic response. Epidermal growth factor has been the focus of mammalian studies of growth factor mediation of the uterine estrogen response. These results demonstrate that in the gecko oviduct, IGF-I appears to be more important than EGF in stimulating oviductal growth and development with respect to endometrial thickness and gland diameter. The in vivo effects of IGF-I on mammalian uteri need to be studied. Additionally, it would be interesting to look at the interactive effects of IGF-I and EGF in

addition to other uterine growth factors in an attempt to construct a complete model of the control of cyclic uterine proliferation and differentiation in vertebrates.

CHAPTER 6

INSULIN-LIKE GROWTH FACTOR-I CONCENTRATION VARIES IN THE EGG OF THE AMERICAN ALLIGATOR, *ALLIGATOR MISSISSIPPIENSIS* DURING EMBRYONIC DEVELOPMENT

Introduction

Little is known concerning the role of polypeptide growth factors during embryonic development, although they have been implicated in the control of both mammalian (Simmen, 1990; Schultz and Heyner, 1993; Shi et al., 1994) and avian (Girbau et al., 1987; Serrano et al., 1990; De Pablo, et al., 1990; Scavo, 1991) embryonic differentiation and growth . A number of mRNAs for growth factors (PDGF, TGFα,TGFβ, IGF-I, and IGF-II) have been detected in the preimplantation mouse embryo (Rappolee, 1988; Rappolee et al., 1990; Harvey and Kaye, 1991) suggesting that these hormones play an important role in early development. For example, TGF-α stimulates the relative rate of protein synthesis in 2-cell stage mouse embryos (Dardik and Schultz, 1991). Additionally, TGFα treatment stimulates a larger percentage of embryos to develop to the blastocyst stage and these blastocysts contain more cells than untreated embryos (Paria and Dey, 1990). A recent study has demonstrated that co-culture of Fallopian epithelial cells with 2-cell stage mouse embryos increased the rate of blastocyst formation, and this effect was significantly decreased when anti-EGF and/or anti-TGFα neutralizing antibodies were added to the culture (Morishige et al., 1993). Both IGF-I and IGF-II appear early in embryogenesis in the chicken (Engstrom, 1987), and the

rat (Beck, 1987). Human embryos also produce both TGF-α and IGF-II (Hemmings, 1992).

The insulin family of growth factors (insulin, IGF-I, and IGF-II) stimulate metabolism and cellular proliferation in preimplantation mouse embryos *in vitro*. For example, insulin stimulates DNA (Heyner et al., 1989) and protein synthesis (Harvey and Kaye, 1988; Rao et al., 1990). Studies have shown that preimplantation mouse embryos cultured with exogenous IGF-I added to the medium exhibit accelerated blastocyst development and increased protein synthesis (Harvey and Kaye, 1992; Rappolee et al., 1992). Heyner et al. (1993) reported an increase in the number of total inner cell mass cells in mouse blastocysts incubated with 40 ng/ml of insulin, IGF-I, or IGF-II, with only insulin resulting in an increase in the total number of cells. Initial studies using reverse transcription-polymerase chain reaction (RT-PCR) suggested that preimplantation mouse embryos do not synthesize their own IGF-I (Heyner et al., 1989; Rappolee et al., 1990). A more recent study by Doherty et al., (1994) , however, detected IGF-I transcripts in the preimplantation mouse embryo by RT-PCR and described the temporal pattern of expression of this transcript from the oocyte to the blastocyst stages. IGF-I transcript levels decrease from the oocyte stage to the 8-cell embryo and then increase from the 8-cell embryo to the blastocyst stage. Maternally derived IGF-I has been detected in the mouse reproductive tract using high-resolution immunoelectron microscopy (Heyner et al., 1993). Messenger RNAs for IGFs are present in pig uterine endometrium (Letcher et al., 1989; Simmen et al., 1990; Lee et al., 1993) as well as bovine uterine endometrium (Geisert et al., 1991) and ovine uterine fluid (Ko et al., 1991).

IGF-I has been identified in the yolk of the chicken egg (Scavo, 1989) and in the albumen of the alligator egg (Guillette and Williams, 1991). The

possible role of IGF-I and insulin during embyogenesis in the chick has been discussed in detail (De Pablo et al., 1990). It has been hypothesized that these two peptides function together to regulate embryonic development by controlling embryonic metabolism (Baroffio et al., 1986), growth (Girbau et al., 1987), and differentiation (Alemany et al., 1989). Insulin-like material has also been detected in the unfertilized eggs of amphibians (De Pablo et al., 1982) and sea urchins (De Pablo et al., 1988). The presence of insulin and IGF-I receptors in early embryos from species as diverse as *Drosophila* (Fernandez-Almonacid and Rosen, 1987), sea urchins (De Pablo et al., 1988), *Xenopus*, chicken (Scavo et al., 1991) and mice (Rappolee et al., 1990) further supports the hypothesis that the insulin family of peptides are essential in embryonic development.

The presence of growth factors such as IGF-I in the egg yolk of chickens and albumen of alligators provides an important model for studying the biological role of growth factors during early embryonic development. Unlike viviparous vertebrates, the oviparous female must provide all of the growth factors, or their mRNAs, required for early development either in the yolk or albumen prior to oviposition. Historically, the egg yolk has been viewed predominantly as an energy source. It has been assumed by vertebrate developmental biologists that because the endocrine system develops relatively late in embryonic life, specific hormones are absent until their endocrine sources undergo organogenesis. However, peptide growth factors and their mRNAs have been found stored in amphibian oocytes (Kimelman et al., 1988; Weeks, et al., 1987). Scavo et al. (1989) detected the presence of an avian IGF-I-like molecule in the yolk of unfertilized chicken eggs. Teleost fish eggs may contain large quantities of thyroid hormone in the yolk (Brown and Bern, 1989). These observations overturn the previous ideas that the

earliest stages of embryogenesis occur in the absence of hormonal influences. Egg albumen has been described principally as a water source and a physical barrier between the developing embryo and the environment; recently, however, a variety of other types of molecules such as vitamin-carriers, anti-bacterial and anti-viral compounds, and growth factors have been identified in albumen (Palmer and Guillette, 1992). The purpose of this study is to examine IGF-I levels in the albumen and yolk of alligator (*Alligator mississippiensis*) eggs at different stages of embryonic development in an attempt to begin to understand the potential role that this growth factor plays during embryogenesis in reptiles.

Materials and Method

Specimens

A mature, gravid female alligator (*A. mississippiensis*) was captured from Lake Griffin in central Florida (permit #W88063) in June, 1993. Within 24 hours of capture, the animal was anesthetized with 20 mg/kg sodium pentobarbital, and the oviducts were surgically removed from the animal. This animal was taken as part of a larger interdisciplinary research project (University of Florida, U.S. Fish and Wildlife Service, Florida Game and Freshwater Fish Commission) on the reproductive biology of the alligator. Twenty-two eggs were removed from the right oviduct and twenty-one eggs from the left oviduct. The eggs were incubated in a tray lined with warm, moist sphagnum moss at 31^{0}C with 95-100% humidity, conditions that produce a sex ratio of approximately 1:1 males and females. Three eggs were removed each week of development. See Table 6-1 for information

concerning week of development versus embryonic stage. Albumen and yolk samples were collected from each egg and stored at -70^0 C.

Table 6-1. Staging of embryonic development in the American alligator, *A. mississippiensis*.

Week of Development	Stage of Development (Approx.)	Age (Range of Days) (Approx.)
1	pre-stage 1	2 days prior to oviposition
2	stage 5	4-5
3	stage 11	11-12
4	stage 15	18-20
5	stage 17	22-23
6	stage 19	27-28
7	stage 21	31-35
8	stage 23	41-45
9	stage 24	46-50
10	stage 25	51-55

Radioimmunoassay

Human recombinant insulin-like growth factor I purchased from Amersham International was used as both a standard and a radioactive tracer (3-[^{125}]iodotyrosyl, Amersham). Rabbit anti-human IGF-I antiserum (Lot #UB3-189), provided by Drs. J.J. Van Wyk and L.L. Underwood through the National Hormone and Pituitary Program was used at a final dilution of 1: 10,000. Bound -free separation was achieved using a secondary antibody reaction (Amerlex-M, Amersham) and magnetic separation. Validation of the assay was accomplished with an internal standard curve and a plasma dilution curve.

Sample extraction was based on Daughaday et al. (1980). Each lyophilized albumen and yolk sample was reconstituted in 5 ml of RIA buffer (200 mg/L protamine sulfate, 4.14 g/L sodium phosphate monobasic, 0.05%

Tween 20, 0.02% sodium azide, 3.72 g/L EDTA). For each sample, four hundred microliters of reconstituted albumen was extracted with 600 µl of acid ethanol (87.5% absolute ethanol, 12.5% 2N hydrochloric acid), vortexed, incubated for 30 minutes at room temperature, and centrifuged at 3,000 g for 10 minutes at 4°C. For the six points in the albumen internal standard curve, 380 µl of the supernatant was aliquotted in duplicate and the samples were spiked with 0, 0.004, 0.015, 0.06, 0.23, or 0.95 ng of IGF-I standard. For the albumen dilution curve, 0, 50, 100, 200, or 400 µl of the supernatant were aliquotted in duplicate from extracted albumen pools. For the yolk internal standard curve, 90ul of the supernatant was aliquotted in duplicate and spiked with 0.014, 0.028, 0.056, 0.113, or 0.225 ng of IGF-I standard. For the yolk dilution curve, 180, 90, 45, and 0 µl of the supernatant were aliquotted in duplicate from extracted yolk pools. For the nine points in the standard curve, 0.009, 0.019, 0.039, 0.078, 0.156, 0.312, 0.625, 1.25, or 2.5 ng of IGF-I standard was added to each tube. Three hundred and eighty microliters of supernatnant from the 400 µl albumen sample which was extracted was used in the assay whereas only 90 µl of supernatant from the 400 µl yolk sample which was extracted was used in the assay.

RIA buffer was added to the standard curve (except the NSB), internal standard curve, plasma dilution, and sample tubes for a total volume of 400µl. RIA buffer (450µl) was added to each of the NSB tubes. IGF-I antibody (50µl) was added to all tubes except the NSB tubes, and labeled IGF-I (50µl) was added to all tubes in addition to two total count tubes. The tubes were vortexed and incubated at 4°C for 24 hours. Then, 300µl of donkey anti-rabbit secondary antibody was added to all tubes except the total count tubes. The tubes were incubated for 10 minutes at room temperature and applied to a magnet for 15 minutes. The supernatant was discarded, the tubes were

drained and the pellets were counted on a Beckman 5500B gamma counter. Extraction efficiency was determined by adding approximately 20,000 cpm iodinated IGF-I to 400µl of pooled albumen or yolk and extracting with 600µl acid-ethanol. Following a 30 minute incubation and 10 minute centrifugation, remaining radioactivity was determined.

Protein Quantification

A modified Lowry procedure (Sigma, Protein Assay Kit, P 5656) was used to quantify protein levels in the albumen and yolk samples. Standard curve tubes were prepared by diluting the protein standard solution to a final volume of 1.0 ml with final concentrations of 50, 100, 200, 400, 600, and 800 µg/ml. A blank tube was prepared with 1.0 ml dH_2O. Albumen samples were aliquotted in 100µl and yolk samples in 25µl volumes and diluted to a final volume of 1.0 ml with dH_2O. One hundred microliters of DOC (1.5 mg/ml aqueous sodium deoxycholate) was added to each tube, and tubes were incubated at room temperature for 10 minutes. One hundred microliters TCA (72% w/v aqueous trichloroacetic acid) was added to each tube. Tubes were centrifuged for 10 minutes to pellet the precipitates. After decanting and blotting supernatants, the pellets were dissolved in 0.5 ml Lowry reagent solution and 0.5 ml dH_2O. Tubes were incubated at room temperature for 20 minutes. One hundred microliters of each standard and sample was transferred to a microplate, and 50µl of Folin & Ciocalteu's Phenol Reagent Working Solution was added. Color was allowed to develop for 30 minutes and absorbances were read at 595 nm. Protein concentration of samples was determined from the standard curve.

Statistical Analysis

For protein quantification, protein concentrations of the albumen and yolk were determined from raw data using Beckman EIA/RIA program. Likewise, IGF-I concentrations were estimated from raw data using the same program. IGF-I levels were reported in pg per μg of protein, log transformed to achieve homogeneity of variance, and analyzed by ANOVA to determine whether significant variation existed in IGF-I concentrations in egg albumen and yolk during different developmental stages (weeks one through ten) in addition to during different developmental periods (early, mid, and late). One-way ANOVA was followed by Fisher's Protected LSD (least-significant difference) test with a significance level set at $p<0.05$.

Results

Radioimmunoassay Validation

Validation results are summarized in Figure 6-1 (albumen) and Figure 6-2 (yolk). The albumen dilution, internal standard, and the human recombinant IGF-I standard curves were parallel, with the test for homogeneity of regression indicating that the curves did not differ. The interassay coefficient of variation was 4.5%. Albumen extraction efficiency was 78%; therefore, final concentrations were adjusted for this 22% loss. The yolk dilution, internal standard, and the human recombinant IGF-I standard curves were also parallel, and no difference was noted when a test for homogeneity of regression was applied. The interassay coefficient of

variation was 1.8%. Yolk extraction efficiency was 79%; therefore, final concentrations were adjusted for this 21% loss.

Insulin-like growth factor-I was found in both the albumen and the yolk of alligator eggs throughout embryonic development. IGF-I concentrations in the albumen (Fig. 6-3A) were highest during the first week of development, and decreased significantly during week 2 ($F=5.387$; $df=8$; $p<0.05$). Levels increased again during weeks 3 and 4 and then gradually dropped off throughout the rest of the developmental period. Albumen IGF-I levels (Fig. 6-4A) were significantly higher during the first two thirds rather than in the last third of the developmental period ($F=7.616$; $df=2$; $p<0.05$). IGF-I in the yolk shows a similar pattern to that seen in albumen IGF-I when examining concentrations versus week of development. Initial yolk IGF-I concentrations (Fig. 6-3B) were high during the first three weeks, dropped significantly during the fourth week, gradually rose again during the fifth and sixth weeks, and declined rapidly during the seventh week and remained low during weeks eight, nine, and ten ($F=2.929$; $df=8$; $p<0.05$). IGF-I concentrations in the yolk (Fig. 6-4B) were highest during the first six weeks of development and decreased significantly during the last four weeks ($F=9.208$; $df=2$; $p<0.05$). Concentrations of IGF-I were approximately seven times higher in albumen than in yolk.

Discussion

The presence of IGF-I in the albumen and yolk of the alligator egg and the fact that it varies throughout embryonic development supports the hypothesis that this growth factor is important in reptilian embryonic development. Insulin-like growth factor I is present in both the albumen and the yolk of the alligator egg. Albumen and yolk IGF-I concentrations varied

during embryonic development, and concentrations in albumen were approximately seven times higher than those in yolk at all stages. In albumen, a relatively high IGF-I level during the first week of development was followed by a significant decline in IGF-I by the second week and then a significant increase by the fourth week. After week 4, IGF-I concentrations steadily declined throughout the rest of development. In the yolk, a similar pattern exists with initially high concentrations during week 1 decreasing significantly by week 4 and rising again by week 6. Following week 6, IGF-I concentrations quickly dropped to very low and then non-detectable levels. One hypothesis, based on these patterns of IGF-I concentrations observed in the albumen and yolk, is that the initial IGF-I in the egg is maternally-derived. The embryo may depletes this supply of IGF-I and may eventually begins to synthesize its own. Consequently, a steady increase in IGF-I is observed, in the albumen during mid embryogenesis. As the embryo continues to develop, the supply of IGF-I is again exhausted prior to hatching. Further research is needed to investigate embryonic IGF-I production.

Experimental evidence for a critical role of polypeptide growth factors during embryonic development is rapidly accumulating . Polypeptide growth factors have been identified in both uterine secretions and in developing embryos suggesting that growth factors play an important role in the regulation of cellular proliferation and differentiation as well as tissue morphogenesis in mammals. These observations combined with the observation that mouse embryos cultured in a simple balanced salt solution with nutrients (in the absence of maternally-derived growth factors) exhibited a significant lag in development as compared with *in vivo* development (Bowman and McLaren, 1970) support the hypothesis that growth factors are critical for early embryonic development.

Similarly, it is evident that non-mammalian forms of these growth factors and their receptors are crucial to non-mammalian embryogenesis (Scavo et al., 1989; Serrano et al., 1990; Scavo et al., 1991; Girbau, 1992). In chickens, it is clear that both the insulin and the IGF-I receptors are expressed at least as early as gastrulation (day 1) and that IGF-I receptors are dominant; additionally, during neurulation (day 2) insulin and IGF-I receptors are abundant in the nervous system. (De Pablo et al., 1990). IGF-I mRNA has been detected in preimplantation mouse embryos (Doherty et al., 1994), and Serrano et al., (1990) have demonstrated using RT-PCR the presence of IGF-I mRNA in whole chick embryos from the late blastula stage through the end of organogenesis (day 8). They also detected IGF-I in the liver, brain, and pancreas during mid-late embryogenesis and at hatching. Interestingly, hepatic IGF-I mRNA levels were barely detectable during mid-late embryogenesis, whereas transcripts in pancreas and brain were readily detectable. Hepatic IGF-I transcripts increased sharply at the peak of postnatal growth (day 50). Circulating IGF-I levels in chicken embryos increase between days 6 and 15 and decrease at later stages of development (Caldes et al., 1990) suggesting that plasma IGF-I originates from extrahepatic sources during prenatal chicken development. In addition to embryonic synthesis of IGF-I, data suggest that IGF-I is stored in the unfertilized chicken egg yolk in concentrations at least as great as those of insulin (Scavo et al., 1989). Growth factors of maternal origin which are incorporated into the egg could play a major role in early growth and differentiation.

The data presented in this study supports this hypothesis that both maternally and embryonically-derived IGF-I are involved in embryogenesis. Insulin-like growth factor I acts on its target cells via endocrine, autocrine, and paracrine pathways. Its presence in alligator egg albumen suggests that

IGF-I is also an important exocrine factor which is actively secreted from the oviduct and incorporated into the albumen as the egg passes along the oviduct. The oviparous vertebrate provides an ideal model for studying the role of growth factors in embryogenesis since all of the maternal information which is necessary for embryonic development must be packaged inside the egg prior to oviposition. The exact mechanism by which IGF-I exerts its biological effects remains to be elucidated. When examining the role of growth factors, it is important to consider not only its function as a single peptide, but also its interactive function with other growth factors. Normal embryonic development depends on a complex interaction between maternally and embryonically-derived growth factors and their receptors on embryonic cells.

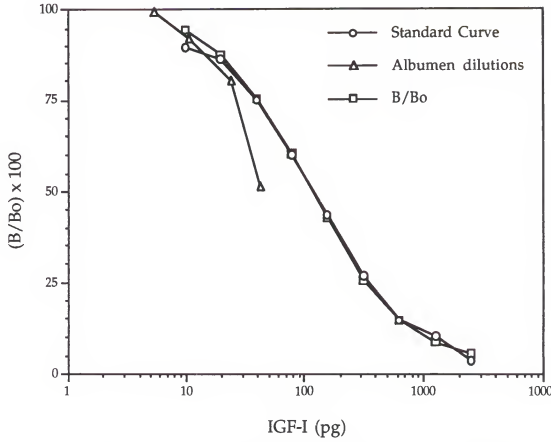


Figure 6-1. Validation for insulin-like growth factor-I radioimmunoassay for the egg albumen of the American alligator, *A. mississippiensis*.

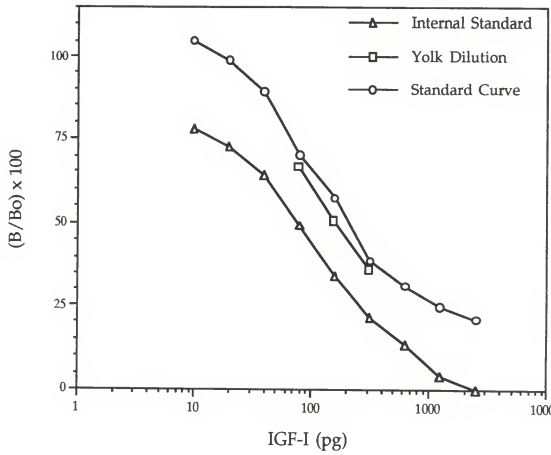


Figure 6-2. Validation for insulin-like growth factor-I radioimmunoassay for the egg yolk of the American alligator, *A. mississippiensis*.

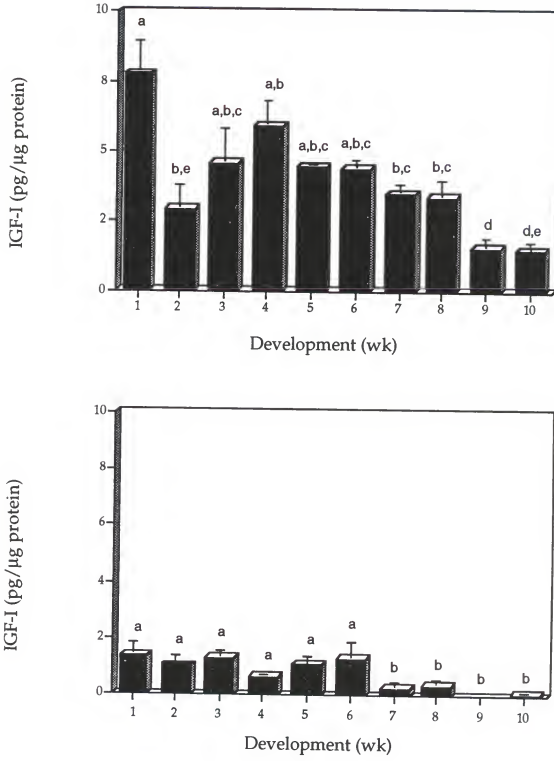


Figure 6-3. Insulin-like growth factor-I concentrations in albumen (top) and yolk (bottom) by week during embryonic development in the American alligator, *A. mississippiensis*.

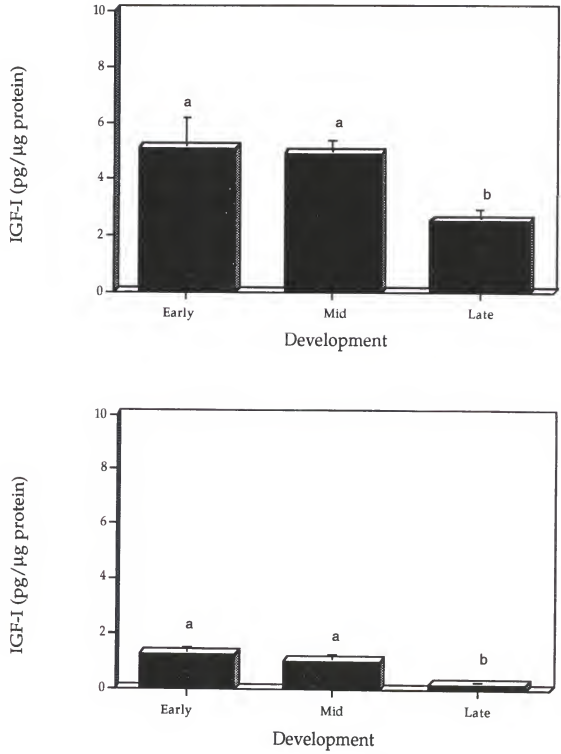


Figure 6-4. Insulin-like growth factor-I concentrations in albumen (top) and yolk (bottom) during early (weeks 1-3), mid (weeks 4-6), and late (weeks 7-10) embryonic development in the American alligator, *A. mississippiensis*.

CHAPTER 7
SUMMARY AND CONCLUSIONS

In mammals, IGF-I and EGF have been implicated as mediators of estradiol-17β action in the reproductive tract. These growth factors also play important roles in the regulation of mammalian embryonic development. The studies presented in this dissertation investigated the presence and functional role of insulin-like growth factor I (IGF-I) and epidermal growth factor (EGF) in the reptilian oviduct. A summary diagram of the functions of IGF-I in reptilian reproductive physiology is presented in Figure 7-1. Data from these studies suggest similar functions for EGF. The results of these studies demonstrate the presence of IGF-I and EGF in reptiles and suggest important roles for these peptides in seasonal oviductal and embryonic development. It appears that IGF-I acts via endocrine, autocrine/paracrine, and exocrine pathways to regulate cellular growth and development. The results of these studies also suggest an autocrine/paracrine role for EGF. Immunocytochemical data demonstrate the presence of these two polypeptide growth factors in the vitellogenic oviduct of the tuatara (*Sphenodon punctatus*), a gopher tortoise (*Gopherus polyphemus*), a lizard (*Sceloporus virgatus*), and the American alligator (*Alligator mississippiensis*). Both growth factors were present in all oviductal regions examined suggesting that they are present in all reptiles and that they play a role in reproduction. In the alligator, patterns of IGF-I and EGF immunoreactivity varied among the three functional oviductal regions (tube, fiber uterus, and calcium uterus) and among the four basic reproductive conditions (nonreproductive, vitellogenic,

gravid, and post-oviposition). Lowest levels of immunoreactivity were detected in the oviduct of the nonreproductive animals. In the vitellogenic, gravid, and post-oviposition animals, positive immunostaining was observed in the apical tips of the epithelial cells, throughout the cytoplasm of the epithelial cells, in the endometrial glands, in the stroma, and in the myometrium.

Insulin-like growth factor I was detected in the plasma of nonreproductive, vitellogenic, gravid, and post-oviposition alligators. This IGF-I represents the endocrine form of the growth factor which is presumably synthesized and released from the liver under the direct control of growth hormone (GH). It is also possible that circulating IGF-I originates from other sources such as the oviduct. Interestingly, nonreproductive animals with low circulating E_2 levels had elevated IGF-I concentrations compared to vitellogenic and post-oviposition animals supporting the hypothesis that E_2 inhibits GH stimulation of hepatic IGF-I synthesis. Insulin-like growth factor I mRNA was identified in the tube, fiber uterus, and calcium uterus from oviducts of nonreproductive, vitellogenic, and post-oviposition animals, the protein transcribed from these transcripts presumably representing the autocrine/paracrine form of IGF-I. Gravid animals exhibited a higher relative abundance of IGF-I mRNA than did nonreproductive or vitellogenic animals.,

The exogenous administration of E_2, IGF-I, or EGF to ovariectomized Mediterranean geckos, *Hemidactylus turcicus*, resulted in stimulation of oviductal growth and development supporting the hypothesis that these growth factors mediate estrogen-induced changes in the reproductive tract. Four morphological parameters were examined: epithelial cell height, endometrial thickness, gland diameter, and myometrial thickness. With

respect to epithelial cell height, IGF-I induced 76% and EGF 65% of the response elicited by E_2. For endometrial thickness, IGF-I induced 71% and EGF 65% of the response elicited by E_2, and for gland diameter, IGF-I induced 83% and EGF 65% of that observed in the E_2 treated oviduct. These data demonstrate an important role for IGF-I and EGF in the gecko oviductal growth and development and suggest these growth factors are critical in the cellular response of the reptilian uterus to E_2 resulting in oviductal proliferation and differentiation. Mammalian studies have focused on the role of EGF in the reproductive tract (Nelson et al., 1990). The data from this study demonstrate that IGF-I could have a more significant effect on oviductal development, specifically on endometrial thickness and gland diameter than does EGF. Consequently, future mammalian studies *in vivo* need to address the role of IGF-I more closely and to focus on the interactive relationships among the various polypeptide growth factors such as IGF-I, EGF, transforming growth factor-α (TGFα), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF) which have been identified in mammalian uterine tissue.

The presence of immunoreactivity in the glands and in the apical tips of the epithelial cells of the uterine tube suggest that IGF-I and EGF could be secreted into the oviductal lumen and incorporated into the albumen as the eggs pass down the oviduct prior to shelling in the uterus. Radioimmunoassay data confirmed that IGF-I is present in the yolk and the albumen of alligator eggs. Levels of IGF-I are highest during early embryonic development and drop significantly prior to hatching. The presence of growth factors such as IGF-I in the egg white provides an important model for studying the biological role of growth factors during early embryonic development. Unlike mammals or other viviparous vertebrates, the

oviparous female must provide all of the growth factors, or their mRNAs, required for early development either in the yolk or albumen prior to oviposition.

McLachlan et al. (1990) proposed a set of four criteria which need to be met in order to establish a biological function for growth factor. First, the endogenous ligand should be present in the appropriate tissue. Second, receptors for the ligand should be localized to the appropriate tissue. Third, administration of exogenous growth factor should produce the appropriate physiological response. Finally, neutralization of the ligand *in situ* should cause reversal of the expected physiological response. Two of these four criteria have been met by IGF-I and EGF in reptiles. First, IGF-I has been identified in the reptilian oviduct by immunocytochemistry and mRNA analysis. It has been identified by radioimmunoassay in the plasma and the egg albumen and yolk of the alligator, *A. mississippiensis*. Therefore, IGF-I is present in the oviduct where it is hypothesized to play a role in seasonal proliferation and differentiation and in the egg where it is hypothesized to be involved in regulation of embryonic development. Epidermal growth factor has been identified immunocytochemically in the reptilian oviduct where it is also hypothesized to play a role in seasonal oviductal development. Administration of exogenous IGF-I and EGF to ovariectomized geckos, *H. turcicus* resulted in growth and differentiation of the oviduct, thus fulfilling a second criterion proposed by McLachlan.

Future studies addressing the presence of IGF-I and EGF receptors need to be carried out. The biological actions of these growth factors are dependent upon the presence of receptors. Additionally, in the case of IGF-I, binding proteins also play a very important role in IGF-I availability to its target tissues. Where are these growth factor receptors located in the reproductive

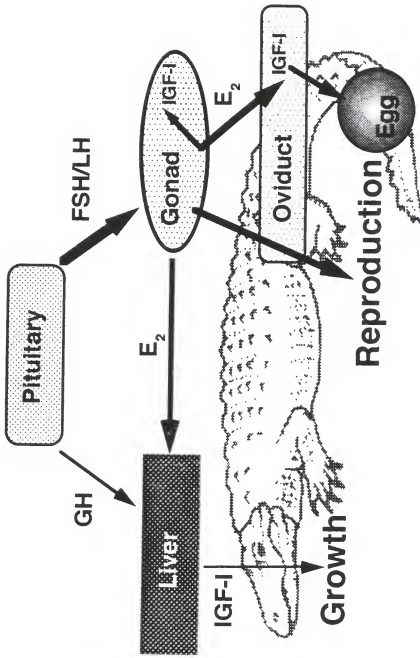
tract? Are they on the epithelial or stromal cells? What hormones regulate the synthesis of these receptors? Are receptors present during early embryonic development? When do they first appear? It would be interesting to study IGF-I and EGF receptors in early embryos and to determine if, as in chickens, the mRNA transcripts for the receptors are present before the mRNA transcripts for the peptides suggesting that maternal growth factors are responsible for eliciting IGF-I and EGF effects before the embryos themselves begin synthesizing the peptides. A final criterion which needs to be addressed is the neutralization of the physiological effects of IGF-I and EGF by antibodies.

Because heterologous antibodies (rabbit anti-human IGF-I and rabbit anti-mouse EGF) were used to localize their respective antigens, conclusions from the immunocytochemical results must be interpreted carefully. Positive immunoreactivity for IGF-I and EGF suggest that IGF-I-like and EGF-like material are present in the oviduct of these representative reptiles. The genes for these growth factors have not been sequenced in reptiles, but the data do suggest a high degree of homology between the reptilian forms of these growth factors and their mammalian counterparts, and the presence of these growth factors in the reptilian oviduct suggests potential physiological roles for IGF-I and EGF in reptilian reproduction.

The presence of IGF-I and EGF in reptiles and possibly throughout the entire vertebrate lineage suggests a key role for these growth factors in the mediation of basic physiological events such as cellular growth, proliferation, and differentiation. The genes for IGF-I have been characterized in several vertebrate species from agnathans to mammals, and the high degree of DNA sequence homology found among these diverse organisms indicate that the IGF-I gene has been well conserved throughout evolution. Evolutionary

constraints have led to the conservation of the IGF-I gene. Protein function depends on the integrity of its tertiary level of organization. Minor changes in amino acid sequences could drastically alter the tertiary structure of the IGF-I peptide and thus, destroy its ability to bind to the IGF-I receptor. Furthermore, binding of IGF-I to its binding proteins is an important aspect of IGF-I physiology that could be altered if changes in the tertiary structure of IGF-I were to occur. Insulin-like growth factor physiology is a complex process involving the interaction of IGF-I and IGF-II with IGF receptors, IGF binding proteins, and other growth factors. The complexity of the IGF system provides many potential sites for control of IGF synthesis and biological activity. Regulation of the IGFs can occur at the level of transcription or it can involve post-translational processing of the peptide. It can involve IGF binding proteins which have been shown to either inhibit or potentiate the effects of the IGFs. Little is known about the comparative aspects of EGF physiology and this is a potential focus for future research. It is likely that EGF is also highly conserved throughout the vertebrate line as a result of the same evolutionary constraints discussed for IGF-I.

Figure 7-1. Functions of IGF-I in reptilian reproductive physiology. The anterior pituitary releases the gonadotropins, FSH and LH. under the control of GnRH from the hypothalmus, and they stimulate follicular growth and steroidogenesis. Developing follicles secrete E_2 which stimulates oviductal growth and differentiation mediated by IGF-I. The oviduct secretes IGF-I which is incorporated into the egg as it passes down the oviduct prior to ovulation. Estradiol-17β also appears to inhibit the release of hepatic IGF-I. Therefore, animals which are nonreproductive and investing most of their energy on somatic growth exhibit elevated circulating IGF-I concentrations. Reproductive animals, on the other hand, which are investing most of their energy on reproduction exhibit decreased plasma IGF-I levels since elevated plasma E_2 inhibits IGF-I production by the liver.


Pituitary
FSH/LH
GH
Gonad
IGF-I
E2
E2
IGF-I
Oviduct
Egg
Liver
IGF-I
Growth
Reproduction

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BIOGRAPHICAL SKETCH

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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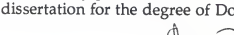

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